INFLUENCE OF FATS AND FATTY ACIDS ON THE CAPILLARIES [1]

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FOUR FIGURES

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The present work was prompted by clinical observations made some years ago (Kramár and Kovács, '39) that the capillary resistance of human subjects, especially of those with allergic manifestations, tends to be higher on a vegetable fat diet than on a diet in which the fat is exclusively of animal origin. A more specific study in which only one type of fat (butter, lard, goose fat, olive oil, coconut oil) was given at a time, also suggested that there was some specific relationship between dietary fats and capillary resistance. As no reference could be found in the literature concerning such a relationship, it was thought desirable to extend these clinical observations to experimental work on animals.

A relationship between capillary resistance and dietary fat suggests two hypothetical explanations: (1) a substance may be present in the dietary fat capable of damaging the capillaries themselves or of interfering with the mechanism regulating their resistance. In this case the presence of this factor in certain types of fat may account for their capacity to diminish capillary resistance; (2) a substance may be present in the dietary fat that is required for the physiologic integrity

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of the capillaries. In such case lack of this dietary substance may account for the decrease in capillary resistance.

In view of our present knowledge of the great difference between various types of fats with reference to the concentration of physiologically active substances such as vitamin A, anti-oxidants, and essential fatty acids, the second hypothesis was chosen for special study.

METHOD OF INVESTIGATION

The experimental animal used was the albino rat. Our first experiments were made to determine the normal capillary resistance of this species. About 100 animals of the Sprague-Dawley strain, of both sexes and of different ages, fed a commercial ration [2] have been observed for over a year to ascertain the normal fluctuations in capillary resistance. The technic used has been described elsewhere (Kramár and Simay-Kramár, '53). At the same time additional experiments were carried out (Kramár and Simay-Kramár, '53) in which various pathologic conditions involving the capillaries were produced, and from these two groups of experiments an over-all picture was obtained of the normal and pathologic variations. These studies led us to the following arbitrary classification of the capillary resistance of the albino rat:

		cm Hg
normal	high	> 70–61
	medium	60–46
	low	45–35
intermediate		34–27
pathologic	moderately decreased	26–11
	very low	10–0

After these preliminary experiments, three groups of weanling albino rats of both sexes, weighing not more than 35 gm, were selected for study. The first group comprised 30 animals (12 males and 18 females). They were fed a synthetic diet

[2] Purina Fox Checkers.

practically devoid of fat and fatty acids but complete in every other respect.[3] The second group contained 24 animals (12 males and 12 females). They received the same diet except that 5% corn oil was substituted for isocaloric amounts of sucrose. In the diet of the third group (14 animals, 8 males and 6 females) 5% methyl stearate was substituted for the corn oil. The capillary resistance was tested every second or third day for a period of at least 150 days, except for 4 animals of the corn oil group and three rats of the methyl stearate group that were not tested during the first 50 days of the experiment, in order to rule out the possible irritating effect of the repeated tests performed upon the skin.

For testing the capillary permeability, the McClure and Aldrich test (McClure and Aldrich, '27) and the intradermal dye-spread method (McMaster, '37; Shanno et al., '47; Shuman and Finestone, '50) were used.[4] In the former, 0.05 ml of Locke solution was injected intradermally and the time of disappearance of the resulting papula was measured. In the dye-spread method, 0.05 ml of fluorescein (1:25000) or of α-azurin (1%) was given intradermally, and the height of the colored papula and its spread were recorded. Because of technical factors the permeability tests could not be performed as frequently as those for capillary resistance, throughout the entire experiments, but had to be limited to a comparison of animals selected from the various groups. Great care was taken in these experiments to use corresponding sectors of the skin of the animals as the sites of injection.

[3] *Composition of diet:* Vitamin test casein 20%; salt mixture, U.S.P. XIV, 4%; sucrose 60%; non-nutritive fiber 16%.

Per 100 gm food: Biotin 15 μg, folic acid 30 μg, thiamine HCl 600 μg, riboflavin 750 μg, calcium pantothenate 1,500 μg, pyridoxine HCl 750 μg, inositol 300 mg, choline chloride 100 mg.

Weekly supplement: α-Tocopherol 3 mg, vitamin A (Aquasol) 100 units, vitamin D (in propylene glycol) 30 units.

[4] These tests, originally designed for rating the cutaneous lymphatic flow, allow inference of capillary permeability only in case the capillary pressure is not changed significantly. The results obtained by these methods must be considered, therefore, with this limitation in mind.

RESULTS

The typical patterns of the weight curve and of the capillary resistance that were found in the three groups of animals are represented in figure 1. The animals fed the fat-deficient basic diet plus 5% corn oil showed a satisfactory gain in weight during the entire period of observation and were perfectly healthy save for two animals that developed pneumonia, one dying on the 45th and the other on the 52nd day.

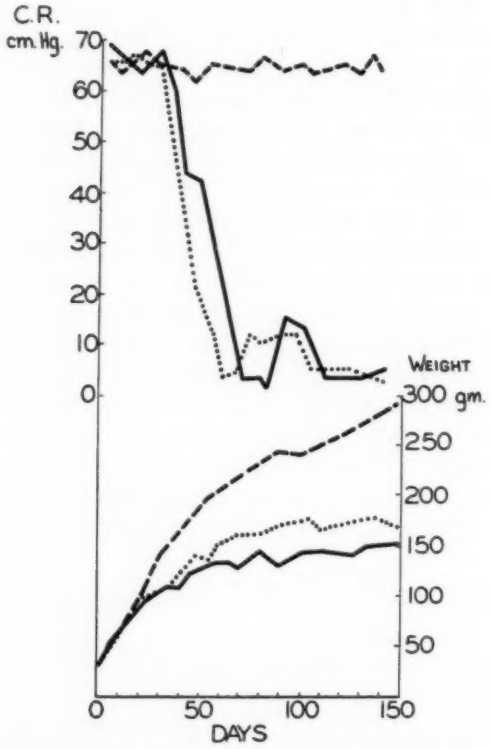


Fig. 1 Typical patterns of capillary resistance and weight curve of rats subsisting on fat-deficient diet (straight line), fat-deficient diet supplemented with 5% methyl stearate (dotted line) and fat-deficient diet supplemented with 5% corn oil (broken line).

Without exception the capillary resistance remained within normal limits. The fluctuations did not exceed $\pm$ 5 cm of the original level found at the beginning of the experiment.

The weight curve of the animals receiving the fat-deficient diet without supplement was similar to that described by Burr and Burr ('29) in their work on fat deficiency in the rat. The first signs of skin manifestation were noted after the 70th day. The fully developed picture of fat deficiency with the characteristic scaling of the paws and tail, however, could not be seen before the 120th day. Necrosis of the tail as described by Burr and Burr ('29) was never encountered.[5] The capillary resistance of these animals revealed a uniform pattern that contrasted very strikingly with that of the control animals fed corn oil. The capillary resistance maintained its original level during the first month of the experiment, but began to decline between the 40th and 50th day. It then dropped precipitously and remained at low levels with fluctuations within the pathologic zone for the rest of the experiment, which continued for several months or until the death of the animals. This drop in the capillary resistance coincided with the flattening of the weight curve. It is highly interesting to note that these two phenomena, the flattening of the weight curve and the drop in the capillary resistance, occurred *before* the skin manifestations developed. It should be stressed that the capillary resistance of the 4 animals that had been spared the testing during the first 50 days of the experiment was found thereafter to follow the general pattern shown by the other members of this group.

The animals receiving the basic fat-free diet plus 5% methyl stearate responded in exactly the same manner as the group subsisting on the fat-free diet without methyl stearate. There was no difference in the clinical picture save for the lower mortality rate. The three animals not tested during the first 50 days also responded in the same manner.

[5] This difference may be due to the fact that the diet of Burr and Burr was not as complete in nutrient factors ('29!) as our experimental diet.

The remarkably uniform character of the capillary resistance of fat-deficient rats raised the question of whether this change in capillary resistance was associated with a similar change in capillary permeability. For this purpose individual animals of the fat-deficient group were compared with those of the control group at a time when the capillary resistance of the former was definitely pathologic, whereas that of the latter was entirely normal. Six animals on the fat-deficient diet were studied for capillary permeability by means of the McClure-Aldrich technique, while 6 animals on the fat-defi-

TABLE 1

Capillary permeability of rats on fat-deficient and control (5% corn oil) diet

McCLURE–ALDRICH TEST[1]

(Time of disappearance in minutes of intradermally injected Locke solution)

DEFICIENT ANIMALS	CONTROL ANIMALS	DIFFERENCE
43	103	60
60	105	45
65	98	33
41	84	43
48	99	41
39	90	51

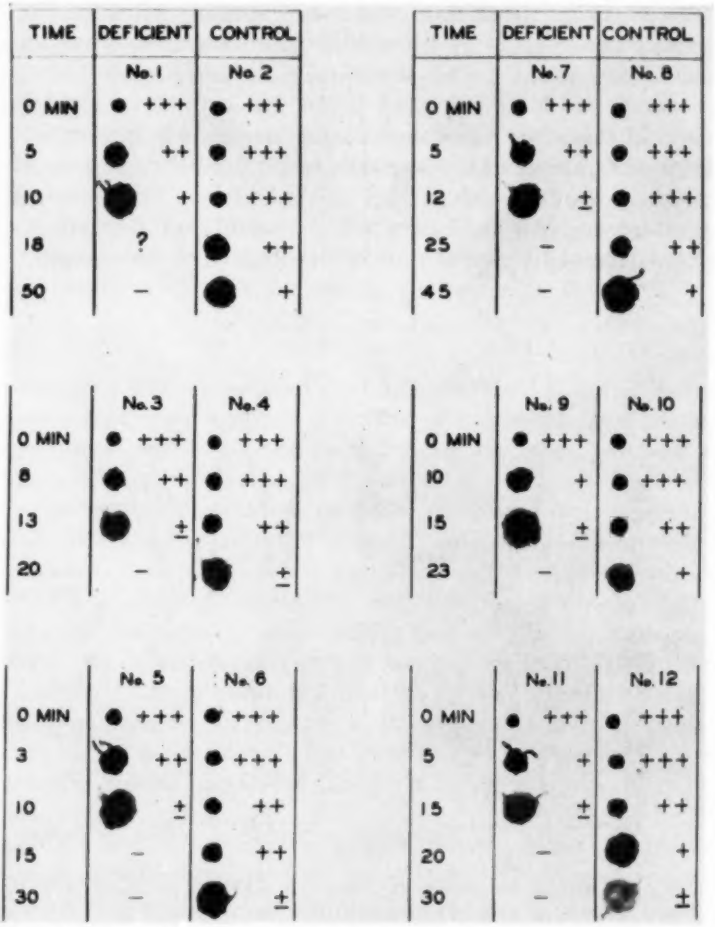
[1] At the time of these tests the capillary resistance of the animals was as follows (in order of sequence): Deficient animals — 5, 7, 4, 4, 8, 2 cm Hg. Control animals — 68, 56, > 70, > 70, 60, 69 cm Hg.

cient diet plus 5% corn oil served as controls (table 1). Another group of 6 fat-deficient animals with 6 controls were studied for capillary permeability by means of the dye-spread technique (table 2).

The results were rather uniform. The time of disappearance of the intradermally administered Locke solution was definitely shorter, and the decrease in the height of the colored papulae and the spread of the dye were markedly faster in the deficient animals than in the controls. These findings indicate, with the limitation mentioned above, an increase in capillary permeability in the fat-deficient animals.

TABLE 2

Capillary permeability of rats on fat-deficient and control (5% corn oil) diet
Intradermal dye-spread method



Size (●) and height (+) of colored papules. At the time of these tests the capillary resistance of the animals was the following (in order of sequence): Deficient animals — 8, 5, 3, 11, 4, 8 cm Hg. Control animals — 62, 70, 50, 52, 68, 47 cm Hg.

After noting a fall in capillary resistance during the fat-deficient regime, an attempt was made to restore it to its normal value. Accordingly 12 rats receiving the fat-deficient diet were given one drop of cottonseed oil daily after the 50th day of the experiment, when their capillary resistance was found consistently to be very low. The effect was exactly the same in all these animals (fig. 2). The capillary resistance began to rise after a few days and returned to normal within two weeks. Though the symptoms of fat deficiency had simultaneously improved, nevertheless this clinical improvement, as evidenced by weight gain and the healing of skin lesions, lagged behind the restoration of the capillary resistance.

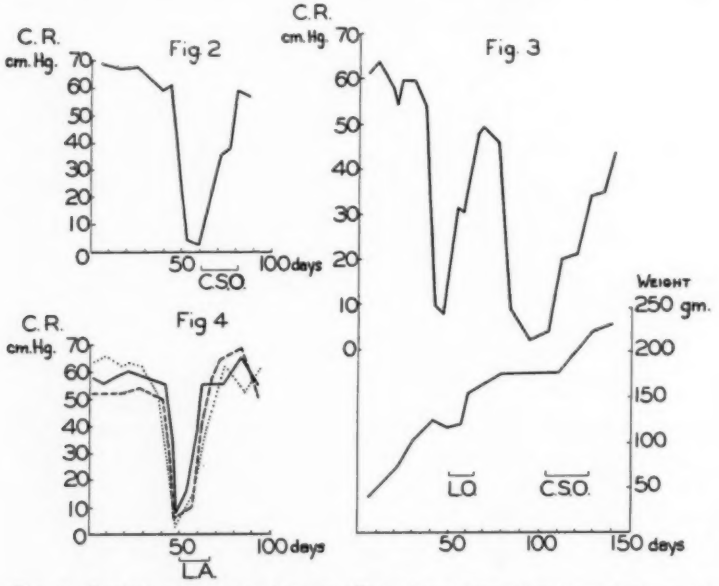


Fig. 2 Typical pattern showing the effect of one drop of cottonseed oil on the capillary resistance of rats subsisting on the fat-deficient diet.

Fig. 3 Typical pattern showing the effect of two drops of linseed oil and one drop of cottonseed oil on the capillary resistance of rats subsisting on the fat-deficient diet.

Fig. 4 Typical pattern showing the effect of one drop of linoleic acid on the capillary resistance of rats subsisting on the fat-deficient diet.

C.S.O.— cottonseed oil; L.O.— linseed oil; L.A.— linoleic acid.

Illustrative data for one experiment out of 6 in which two drops of linseed oil were given daily are presented in figure 3. In these experiments the oil was discontinued after 12 days. The capillary resistance remained high for a week and then dropped even lower than it was before the oil was administered. Cottonseed oil given thereafter resulted again in a prompt increase.

In view of the high content of essential fatty acids in these oils, we resorted in the next experiment to the administration of linoleic acid. One drop (27 mg) of linoleic acid given daily to three rats on the fat-free diet caused an increase in capillary resistance in 4 days and complete restoration of the resistance to normal values within 10 days (fig. 4).

DISCUSSION

Our observations indicate that a fat-deficient diet, lacking in essential fatty acids, may cause the skin capillaries of immature albino rats to become more fragile and more permeable. Our observations also suggest that fats, and presumably essential fatty acids, are necessary for the physiologic integrity of the skin capillaries of the immature albino rat. The authors are conscious of the possible significance of the above findings in both physiology and pathology, but they are also fully aware of the dangers and pitfalls that may arise from any further early conclusions and generalizations. It is felt that a number of open questions must be answered before an attempt is made to evaluate these findings on a broader basis, or to correlate them with the clinical observation that gave incentive to the present work. Only a few points should be mentioned at this time.

Is the ability of linoleic acid to restore the capillaries shared by other members of the essential fatty acid group? If so, is this ability limited to the essential fatty acids or may other substances produce the same effect? Can the capillary phenomenon described above be encountered with other species than the rat? Does it occur in mature animals as well as in immature animals? Is the typical capillary response

during fat deficiency a general phenomenon involving all the capillaries of the body, or is it limited to the capillaries of the skin? This last question requires some discussion.

Since publication of the findings of Hansen and Burr ('46), it has been a generally accepted fact that fats, in particular essential fatty acids, have an important role in the physiologic maintenance of the skin. Fat-deficient diets, or, more specifically, lack of essential fatty acids in the diet, may under certain conditions give rise to visible pathologic changes in the skin, such as hyperemia, dryness and scaling. This has been demonstrated in the rat (Burr and Burr, '29), in the mouse (White et al., '43) and in the dog (Hansen and Wiese, '43, '51). The clinical observations of Hansen ('33a, b, '37) seem to indicate that the human race is no exception to this rule, and that some relationship may exist between fats and oils and allergic skin manifestations. Histologic studies on the rat (Williamson, '41) and on puppies (Hansen et al., '51) clearly demonstrate that there is a profound change in the skin structure during fat deficiency. In their very meticulous study, Hansen and his associates found that the endothel cells of the skin capillaries may show a definite swelling.

Though the subjects of the above study (Hansen and Wiese, '51) were in a far advanced stage of the deficiency, whereas the capillary resistance in our animals was found abnormal *before* any skin lesions occurred, the possibility cannot be disregarded that the fall in capillary resistance may represent the first step in, or the first sign of, a local derangement, which takes place in or around the capillary wall, or both, as part of a pathologic process developing in the skin. Only further studies can demonstrate whether the typical changes in capillary resistance caused by fat deficiency are limited to the skin capillaries or whether the changes represent a general phenomenon involving all capillaries.

It is believed that even when alterations in capillary resistance are limited to the skin, the observations presented in this paper may have some clinical significance. Testing for capillary resistance in the human being is performed on the

skin. For proper evaluation of our finding it is indispensable to consider the factors known to influence capillary resistance. Among these factors may be mentioned temperature, time of day, season, site of testing, the menstrual cycle, and the diet, so far as certain vitamins are concerned. Another factor, hitherto not considered, must now be added. This new factor is dietary fat.

SUMMARY

1. The capillary resistance and capillary permeability of the immature albino rat were studied under various dietary conditions. When the animals were fed a synthetic diet practically devoid of fat and fatty acids, or a synthetic diet containing 5% methyl stearate, the capillary resistance began to decline about the 50th day of the experiment and dropped to pathologically low levels. The difference amounted to 35 to 65 cm Hg,, depending on the initial level (35 to $>$ 70 cm). If the same basic diet contained 5% corn oil, the capillary resistance remained normal.

2. This drop in capillary resistance occurred simultaneously with the flattening of the weight curve, but before the characteristic manifestations of fat deficiency had developed.

3. Among the manifestations of fat deficiency no caudal necrosis was ever encountered.

4. A comparison between the capillary permeability of the animals showing abnormally low capillary resistance and that of the animals with normal capillary resistance indicated that the decrease in the capillary resistance of rats fed a fat-deficient diet or a diet containing methyl stearate was associated with an increase in the capillary permeability.

5. The low capillary resistance of fat-deficient rats was promptly restored by minute amounts of linseed oil or cottonseed oil or pure linoleic acid.

6. In testing for capillary resistance in the human being, a new factor influencing this phenomenon must be taken into consideration. This new factor involves dietary fat.

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EFFECT OF DIETARY PROTEIN AND VITAMIN B_6 ON HEPATIC THIONASE ACTIVITY [1, 2]

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FOUR FIGURES

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It has been observed that weanling rats receiving a vitamin B_6-deficient diet containing 30% casein manifest deficiency symptoms in about one-half the time required to obtain comparable symptoms in rats maintained on a 15% casein diet (Cerecedo and Foy, '44). A more rapid rate of depletion also occurs when either 0.31% methionine, 0.34% methionine sulfoxide, or 0.5% cystine is added to a vitamin B_6-free diet containing 15% casein (Cerecedo, Foy and DeRenzo, '48). Homocystine has been shown to exert a similar effect when added to vitamin B_6-deficient diets (Cerecedo and DeRenzo, '50). However, there is little evidence that other amino acids exert this effect (Cerecedo and DeRenzo, '50).

Recently it was reported (Braunshtein and Azarch, '50) that the activity of cysteine desulfhydrase was depressed in the livers of vitamin B_6-deprived rats but returned to normal following pyridoxine supplementation.

Binkley and Okeson ('50) have shown that the cleavage of cystathionine to yield cysteine and the cleavage of the

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[2] Data taken from a thesis presented by Robert Q. Thompson to the Graduate School of The Pennsylvania State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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sulfhydryl group from cysteine which results in the formation of hydrogen sulfide are catalyzed by similar if not identical enzymes. The term "thionase" was introduced to designate this enzyme or enzymes exhibiting activity toward thioethers (Binkley, '50). It was subsequently shown that pyridoxal phosphate not only served as a coenzyme for the cleavage enzyme (Binkley and Christensen, '51), but also was required by the enzyme involved in the synthesis of cystathionine (Binkley, Christensen and Jensen, '52). Binkley et al. ('52) observed that the activity of the enzymes concerned with the transfer of sulfur is depressed in the livers of mature rats maintained on a vitamin B_6-deficient diet for 41 days. A marked increase in enzyme activity occurred following the addition of pyridoxal phosphate to enzyme preparations prepared from the livers of vitamin B_6-deprived rats. Pyridoxal phosphate also functions as a coenzyme for bacterial cysteine desulfhydrase (Delwiche, '51; Kallio, '51).

On the basis of the published data there appears to be at least one enzymatic reaction involved in the metabolism of the thio amino acids which requires pyridoxal phosphate as a coenzyme. This suggests that the addition of thio amino acids to a diet, either as a protein or as the free amino acids, will result in an increase in the requirement for pyridoxal. This increased requirement for pyridoxal in rats receiving a suboptimum amount of vitamin B_6 may result in a more rapid depletion of the vitamin B_6 reserve. It is also conceivable that in the absence of sufficient vitamin B_6, products are formed, either by incomplete thio amino acid metabolism or by alternate metabolic pathways, which are responsible for the vitamin B_6 deficiency syndrome (DeBey, Snell and Baumann, '52). It therefore seemed desirable to determine the effects of pyridoxine supplementation and the effects of diets which varied in their content of casein or of thio amino acids on the cysteine desulfhydrase (thionase) activity in the livers of growing rats.

EXPERIMENTAL

Weanling albino, black and piebald rats, weighing between 40 and 50 gm, were housed individually in all-metal cages provided with raised screen floors. Food and water were given to the test animals ad libitum. The composition of the vitamin B_6-deficient diets employed in this investigation is presented in table 1. The amount of free thio amino acids added to diet 3 was approximately equivalent to the amount present in diet 1, which contained 20% casein. A supplement of crystalline B vitamins (other than pyridoxine) diluted with cornstarch was added to each diet at a level of 1%. This

TABLE 1

Composition of the vitamin B_6-deficient diets

INGREDIENTS	DIET		
	1	2	3
	gm	*gm*	*gm*
Purified casein	20	40	20
Sucrose (commercial)	67	47	66.22
Hydrogenated vegetable oil (Crisco)	5	5	5
Salt mixture (U.S.P.)	4	4	4
Cellu flour	2	2	2
Cod liver oil (medicinal grade)	1	1	1
Vitamin B mix	1	1	1
DL-Methionine	..	..	0.7
L-Cystine	..	..	0.08

supplement furnished the following amounts of vitamins, in milligrams per kilogram of diet: thiamine 2.5, riboflavin 4.0, calcium pantothenate 20.0, *p*-aminobenzoic acid 4.0, niacin 50.0, inositol 1.0, and choline chloride 500. The cod liver oil used in the diets contained 1,800 U.S.P units of vitamin A and 175 U.S.P. units of vitamin D per gram. In addition to the studies with these diets, the rats used in one experiment were maintained on a breeding colony ration (stock diet). A supplement of 50 μg of pyridoxine hydrochloride contained in 0.5 ml of a 5% sucrose solution was placed in a shallow porcelain fraction cup and fed on alternate days to the experimental control animals.

The weanling rats were distributed, taking into consideration litter, sex and initial weight, into experimental groups consisting of three animals, and frequently the experiments were repeated using similar groups of rats. For each vitamin B_6-deprived group there was a comparable control group of animals which received a supplement of 50 μg of pyridoxine on alternate days during the experimental period. At the end of the respective experimental periods — namely, zero, three, 7, 14, 21, 28, and 35 days — the rats from the vitamin B_6-deprived and control groups, respectively, were anesthetized and bled by severing the carotid arteries. The livers were removed, blotted dry, weighed and individually homogenized for three minutes in a combustion-type test tube (15 × 1.5 cm) with a volume of cold physiological saline approximately equivalent to twice the weight of the liver. A stainless steel homogenizer was employed. After centrifuging the homogenate for 30 minutes and diluting the saline liver extract, if necessary, to a volume containing an enzyme activity suitable for assay, the cysteine desulfhydrase (thionase) activity of the extract was determined in the Warburg apparatus.

In the main compartment of a conventional Warburg flask were placed 2 ml of the liver extract and 1 ml of a 0.1 M phosphate buffer having a pH of 7.2. The side arm of the flask contained 2×10^{-5} M of cysteine hydrochloride in 0.5 ml of a neutralized aqueous solution. A short piece of glass tubing containing an insert of folded filter paper (7 × 5 cm) was placed in the center well of the flask. The filter paper had been saturated previously with a 10% solution of $CdCl_2$. Duplicate flasks were prepared for each liver extract. After flushing the manometer and attached flask with nitrogen for 15 min., the substrate was tipped into the flask and incubated for two hours at 37°C., accompanied by shaking. The evolved H_2S was precipitated quantitatively as CdS on the filter-paper insert in the center well. At the end of the reaction period, the filter-paper insert was removed from the Warburg flask and the precipitated sulfide was determined iodometrically in

the usual manner. The amount of H_2S produced per gram of tissue was calculated for each liver.

In those cases in which the effects of the addition of pyridoxal phosphate [4] to liver extracts from vitamin B_6-deprived rats were studied, the experimental procedure was modified in the following manner. The saline extracts from the livers of several vitamin B_6-deprived rats were pooled, and aliquots of the pooled extracts were incubated for 30 min. at 37°C. with varying amounts of pyridoxal phosphate prior to determination of cysteine desulfhydrase (thionase) activity.

Another phase of the investigation consisted of determining the thionase activity of extracts of livers from rats which had been maintained on vitamin B_6-deficient diets and subsequently supplemented with pyridoxine.

RESULTS AND DISCUSSION

It is to be noted from the data presented (table 2, fig. 1) that the extracts from the livers of rats which had received the breeding (stock) ration during the first two weeks of the experiment showed a marked increase in the amount of H_2S produced. Hydrogen sulfide production reached a maximum at 21 days and thereafter decreased. During an earlier exploratory experiment, the amount of H_2S produced averaged 320 μg per gram of liver for rats maintained on a commercial chow ration for 300 days. These data suggest that maximum thionase activity occurs during the period of most rapid animal growth and then decreases with increase in age.

In those experiments in which pyridoxine-supplemented rats were maintained on diets containing 20% casein, 40% casein, or 20% casein supplemented with DL-methionine and L-cystine, respectively (table 2, figs. 2 and 3), a decrease in the production of H_2S occurred during the first week of the experiment. However, a progressive increase in the thionase activity was observed during the succeeding experimental peri-

[4] The authors are indebted to Dr. Karl Folkers of Merck and Co., who kindly furnished the pyridoxal phosphate and pyridoxamine phosphate used in these studies.

TABLE 2

Effect of feeding supplementary pyridoxine to rats on weight gain, food intake, liver weight and hepatic hydrogen sulfide production

DAYS	NO. RATS		AVE. WT. GAIN	AVE. FOOD INTAKE	AVE. LIVER WT.	AVE. H_2S/GM LIVER
	M	*F*	*gm*	*gm*	*gm*	*µg*
			ALBINO STRAIN — BREEDING DIET			
7	1	2	22	58	2.69	361
14	1	2	48	131	3.86	444
21	1	2	71	228	5.08	458
28	1	2	90	326	6.50	425
			ALBINO STRAIN — 20% CASEIN DIET — 0 µg B_6·HCl			
3	3	3	9	16	2.96	42
7	4	2	16	36	3.37	14
14	4	2	27	73	3.62	17
21	2	1	36	112	4.13	14
28	2	1	36	153	4.22	5
35	2	1	34	207	4.31	3
			ALBINO STRAIN — 20% CASEIN DIET — 25 µg B_6·HCl			
0	3	3	..	..	2.08	221
3	4	2	13	19	3.43	154
7	3	3	27	49	4.49	104
14	4	2	57	113	5.56	139
21	2	1	83	189	7.15	150
28	4	2	86	245	7.21	191
35	2	1	122	375	9.75	175
			PIEBALD STRAIN — 20% CASEIN DIET — 0 µg B_6·HCl			
7	2	1	18	36	3.61	21
14	2	1	25	72	3.64	16
21	1	2	23	106	3.28	14
			PIEBALD STRAIN — 20% CASEIN DIET — 25 µg B_6·HCl			
0	5	2	..	..	1.96	151
7	5	1	29	52	3.95	82
14	4	2	53	115	4.91	124
21	6	1	63	180	5.50	134
28	2	1	80	276	6.37	155
			ALBINO STRAIN — 40% CASEIN DIET — 0 µg B_6·HCl			
3	5	1	3	12	2.31	38
7	5	1	12	31	3.23	9
14	2	1	22	68	3.59	3
21	2	1	32	115	4.63	3

TABLE 2 *(continued)*

DAYS	NO. RATS		AVE. WT. GAIN	AVE. FOOD INTAKE	AVE. LIVER WT.	AVE. H_2S/GM LIVER
	M	*F*	*gm*	*gm*	*gm*	*µg*
	ALBINO STRAIN — 40% CASEIN DIET — 25 µg B_6·HCl					
0	3	3	..	..	2.08	221
3	5	1	12	18	3.13	174
7	5	1	30	48	4.36	167
14	2	1	52	93	5.31	191
21	2	1	88	176	7.61	212
28	2	1	100	242	7.65	255
ALBINO STRAIN — 20% CASEIN DIET — THIO AMINO ACIDS — 0 µg B_6·HCl						
3	1	2	2	16	2.39	46
7	2	1	10	34	2.56	18
14	1	1	20	76	3.72	10
21	3	0	20	96	3.31	8
ALBINO STRAIN — 20% CASEIN DIET — THIO AMINO ACIDS — 25 µg B_6·HCl						
0	2	3	..	..	1.83	184
3	1	2	7	18	3.22	169
7	1	2	27	51	4.34	114
14	1	2	47	114	5.49	119
21	2	1	70	166	7.01	138
28	2	1	87	245	7.45	183

ods. The amount of H_2S produced was considerably greater for liver extracts from pyridoxine-supplemented rats which had received the 40% casein diet than for extracts from animals maintained on the other two semisynthetic diets during comparable experimental periods. However, in no instance did the production of H_2S approach the amount produced by the livers of rats which had received the breeding ration. The reduction in thionase activity observed during the first week of the experiment in the livers of rats receiving the casein diets may have been due to metabolism changes resulting from the transfer of the weanling young from a diet consisting primarily of milk to a semisynthetic diet. The fact that the greatest range in thionase activity values observed during any specific experimental period occurred in animals taken directly from the breeding colony would tend to support this

explanation. Since the ratio of the amount of breeding ration to milk consumed by young rats before weaning would undoubtedly vary, a considerable range in hepatic enzyme activity might be anticipated.

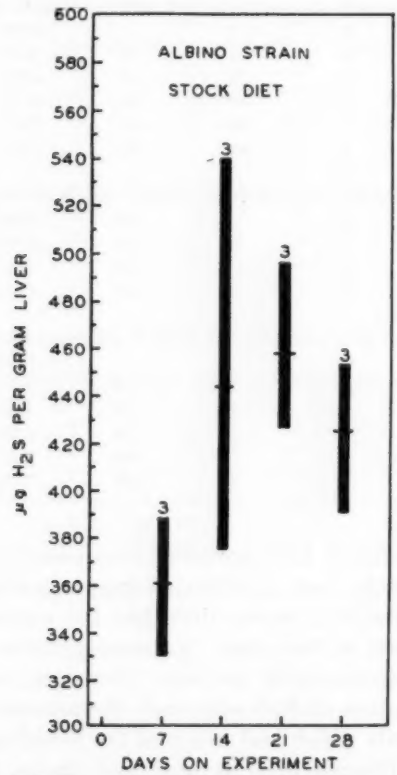

Fig. 1 Hepatic hydrogen sulfide production. The figure at the top of each bar indicates number of livers assayed; the transverse line indicates the average H_2S production.

It is of interest to note that the production of H_2S by the pyridoxine-supplemented rats receiving the 20% casein diet supplemented with DL-methionine and L-cystine was comparable to the amount of H_2S produced by rats receiving the un-

supplemented 20% casein diet. It was anticipated that, owing to the inclusion of thio amino acids in this diet, the hepatic production of H_2S might be equal to that of the rats receiving the 40% casein diet.

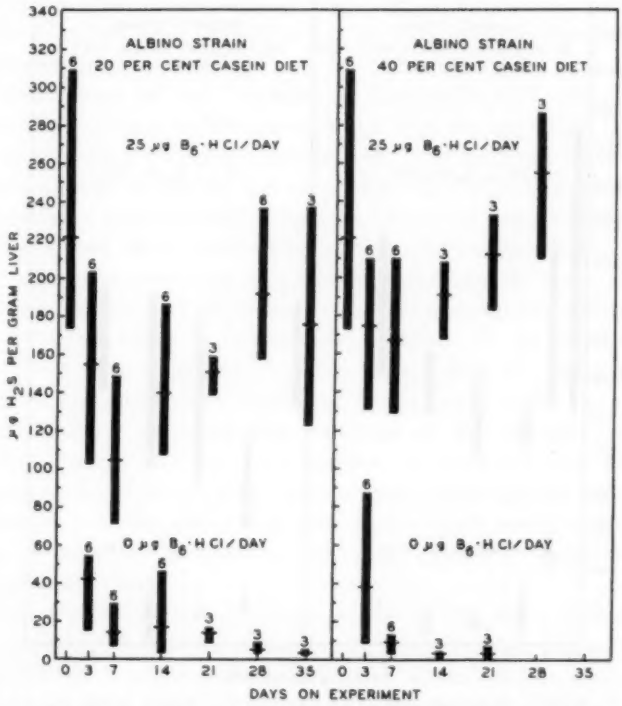
ALBINO STRAIN
20 PER CENT CASEIN DIET
25 μg B6·HCl/DAY
0 μg B6·HCl/DAY
ALBINO STRAIN
40 PER CENT CASEIN DIET
25 μg B6·HCl/DAY
0 μg B6·HCl/DAY
μg H2S PER GRAM LIVER
DAYS ON EXPERIMENT
0 3 7 14 21 28 35
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340

Fig. 2 Hepatic hydrogen sulfide production. The figure at the top of each bar indicates number of livers assayed; the transverse line indicates the average H_2S production.

While only slight differences were observed in the production of H_2S by the livers of the piebald and of the albino rats which had subsisted on the 20% casein diet supplemented with pyrodixine, there was a tendency toward lower thionase activity in the livers from the former strain of rats.

The food intakes, weight gains and liver weights of albino rats which had been maintained on the semisynthetic diets supplemented with pyridoxine were comparable for similar experimental periods. However, the piebald rats tended to

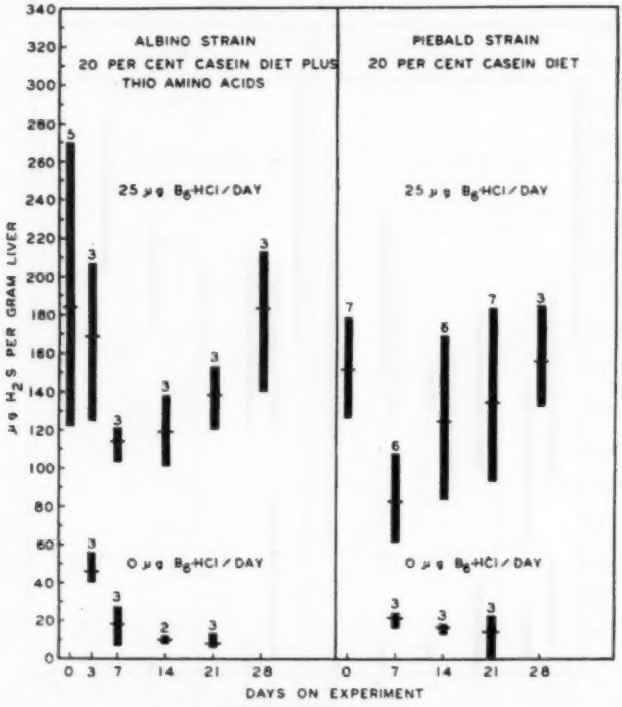
ALBINO STRAIN
20 PER CENT CASEIN DIET PLUS THIO AMINO ACIDS
PIEBALD STRAIN
20 PER CENT CASEIN DIET
25 μg B6·HCl / DAY
0 μg B6·HCl / DAY
μg H2S PER GRAM LIVER
DAYS ON EXPERIMENT
0 3 7 14 21 28
0 7 14 21 28

Fig. 3 Hepatic hydrogen sulfide production. The figure at the top of each bar indicates number of livers assayed; the transverse line indicates the average H_2S production.

gain less weight and had smaller livers during the latter two weeks of the experiment, as compared to the albino rats. The albino rats maintained on the breeding ration, although comparable to the albino rats maintained on the semisynthetic diets with respect to weight gained, did consume more food and had smaller livers.

An interesting observation was the close agreement in the range of liver thionase activity values obtained for the rats comprising the several experimental groups receiving the same dietary treatment. Although the sex of the rat appeared to have little influence on the amount of H_2S produced, rats from the same litter receiving similar experimental treatment showed much less variation in hepatic H_2S production than did non-litter mates.

The livers of the vitamin B_6-deprived rats exhibited a marked decrease in the amount of H_2S produced at the end of a three-day experimental period, as compared to the control animals receiving the respective diet supplemented with pyridoxine (table 2, figs. 2 and 3). After 21 days the thionase activity had been reduced to a negligible value in the livers of vitamin B_6-deprived rats subsisting on the 20% casein diet. This is in marked contrast to the time required for a comparable reduction in the thionase activity of the vitamin B_6-deprived rats receiving the 40% casein diet (7 days) or the 20% casein diet supplemented with thio amino acids (14 days), respectively. Although the addition of thio amino acids to a 20% casein diet did not appear to influence the hepatic thionase activity in the pyridoxine-supplemented rats, the thionase activity decreased at a more rapid rate in the vitamin B_6-deficient animals as the result of the thio amino acid supplementation.

The B_6-deprived rats which received the 40% casein diet or the 20% casein diet supplemented with thio amino acids manifested a more severe deficiency at an earlier date than did those maintained on the 20% casein diet. Fatty livers were frequently observed in the vitamin B_6-deficient rats.

A linear response in the production of H_2S was obtained upon the addition of graduated amounts of pyridoxal phosphate to vitamin B_6-deficient liver extracts (fig. 4). Approximately 14 μg pyridoxal phosphate were required to obtain maximum thionase activity in liver extracts from rats deprived of vitamin B_6 for more than 7 days. It was of interest to note that about one-half as much pyridoxal phosphate was required

to obtain maximum thionase activity in extracts of livers from rats which had been depleted of vitamin B_6 for only 7 days. This observation supports the initial finding that a very rapid reduction in thionase activity occurs in rats maintained on a vitamin B_6-free diet containing 40% casein (fig. 2). It was observed that increased production of H_2S occurred in ex-

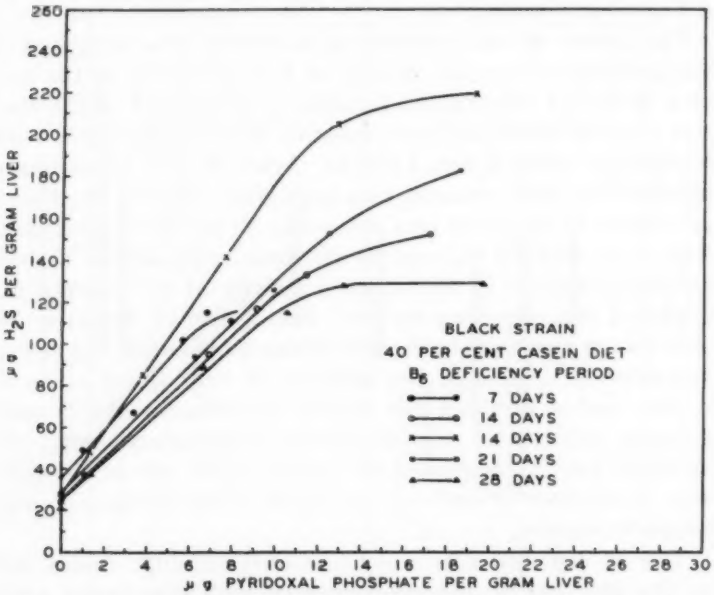

Fig. 4 Effect of pyridoxal phosphate on the *in vitro* stimulation of hydrogen sulfide production by liver extracts from vitamin B_6-deprived rats.

tracts of liver obtained from control animals when pyridoxal phosphate was incubated with the extracts prior to the determination of thionase activity. Pyridoxine, pyridoxal, pyridoxamine and pyridoxamine phosphate [5] were found to be ineffective in restoring thionase activity when added to extracts of livers from vitamin B_6-deficient rats.

[5] See footnote 4, p. 165.

The data obtained in this investigation indicate that vitamin B_6 in the form of pyridoxal phosphate is essential for the functioning of the enzyme, cysteine desulfhydrase (thionase). The fact that a marked decrease in hepatic thionase activity occurred during the first three days of B_6-deprivation suggests that tissue vitamin B_6 is not readily utilized by the rat.

These studies demonstrate that the activity of thionase is readily restored in the livers of vitamin B_6-deprived rats by pyridoxine supplementation. Furthermore, it is shown that the addition of pyridoxal phosphate to liver extracts from B_6-deprived rats also restores the thionase activity.

In addition, the results of these studies indicate that an increase in thio amino acid intake by rats receiving vitamin B_6-deficient diets results in a more rapid utilization of the animal's pyridoxal phosphate reserve. Consequently, rats receiving vitamin B_6-deficient rations containing a high proportion of casein or of thio amino acids manifest a vitamin B_6 deficiency syndrome at an earlier date than do animals fed other diets.

SUMMARY

A study has been made of the effects of diets which varied in their content of casein or of thio amino acids and of pyridoxine on the hepatic thionase activity of growing rats. In addition, the hepatic thionase activity of rats which had been depleted of their vitamin B_6 reserve and subsequently fed pyridoxine has been determined. Furthermore, the effectiveness of pyridoxal phosphate in restoring thionase activity to liver extracts obtained from vitamin B_6-deprived rats was investigated. The results of these studies are summarized as follows:

1. Growing rats maintained on a breeding colony ration exhibited increasing hepatic thionase activity during the first three weeks of the experimental period, followed by a subsequent decrease in thionase activity.

2. Rats maintained on the semisynthetic diets supplemented with pyridoxine also showed increasing hepatic thionase ac-

tivity during the experimental period following one week of adaptation to the regimen.

3. A marked and rapid decrease in hepatic thionase activity was observed in rats maintained on vitamin B_6-deficient diets. The addition of casein or thio amino acids to a vitamin B_6-deficient diet containing 20% casein resulted in a more rapid decrease of hepatic thionase activity.

4. Rats maintained on diets deficient in vitamin B_6 and containing 40% casein or added thio amino acids manifested vitamin B_6 deficiency symptoms in a much shorter period of time than did rats maintained on a 20% casein regimen.

5. Rats which were deprived of vitamin B_6 for 21 days showed a rapid restoration of hepatic thionase activity following pyridoxine supplementation.

6. The addition of pyridoxal phosphate to liver extracts obtained from vitamin B_6-deprived rats restored hepatic thionase activity.

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THE INFLUENCE OF ALPHA-TOCOPHEROL UPON THE UTILIZATION OF CAROTENE AND VITAMIN A

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TWO FIGURES

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Moore ('40) was the first to report the effect of vitamin E deficiency on the vitamin A reserves of the rat. He found an increase in the amount of vitamin A stored in the livers of the supplemented animals amounting to some 10 times that of the non-supplemented group. Further work by Davies and Moore ('41) showed the effect of vitamin E in increasing the time required for depletion of liver stores of vitamin A. These authors and Quackenbush et al. ('42) concluded that tocopherols act as antioxidants in the gastrointestinal tract. Sherman ('41) believed that in the absence of tocopherols there is a physiological antagonism between the unsaturated fatty acids and carotene which results in an inefficient use of the latter, probably through enzymatic destruction.

Hickman and the group at Distillation Products, Inc. (Harris et al., '44, '47; Hickman et al., '44) found that tocopherols enhanced the growth-promoting powers of vitamin A alcohol, vitamin A acetate and the standard U.S.P. reference oil. They also showed that it took more time to deplete animals of vitamin A if tocopherols were given and that animals took longer to die after vitamin A supplementation ceased if tocopherols were present. These workers believed that carotene and tocopherols are synergistic in roughly proportional amounts.

Lemley et al. ('47) confirmed the beneficial effect of 0.3 mg daily of tocopherol on liver storage of vitamin A if 100 I.U.

daily were give for three months, but noted much less effect if the time or the dose was doubled. Johnson and Baumann ('48a) however, found evidence of a depressing effect of high levels of tocopherol on the liver stores and the growth of their carotene-fed animals. Koehn ('48), using a low-fat diet and growth as criterion, reported as good response by rats to 1.0 μg β-carotene daily as to the same amount of vitamin A when 1.0 mg α-tocopherol was given daily. Smaller doses were not tried. Burns, Hauge and Quackenbush ('51) followed this by a careful study of the growth of depleted rats given 1.0 μg of vitamin A acetate or of β-carotene with 0, 0.5, 1.0 or 2.0 mg α-tocopherol daily. At the 1-mg level the growth response was as good with the carotene as with the vitamin A. The tocopherol appeared to have no significant effect upon the vitamin A-fed animals. In some cases Tween 40 was used to produce an aqueous dispersion of the vitamin A and carotene but this was followed by a depression of growth in the case of vitamin A and no change in the case of β-carotene. The conclusion drawn from both these studies was that pure β-carotene and vitamin A are of equal biological potency when an adequate amount of α-tocopherol is given with them. It should be noted that in these experiments only the minimum levels of vitamin A and carotene were used and that utilization and storage of optimum doses may involve different mechanisms.

The object of the experiments here reported was to observe the effect of a fixed dose of tocopherol upon the liver storage of vitamin A with rather widely varying daily intakes of vitamin A and carotene, the latter in aqueous as well as in oily solution. The rate of appearance of vitamin A in the intestine and liver when aqueous solutions of carotene were used was also noted and compared with that previously reported (Mattson et al., '47) for oily solutions. At the same time an attempt was made to determine the critical daily dosage of vitamin A and carotene at which cumulative liver storage of the vitamin A may be expected to occur. This may be a measure of the utilization of vitamin A by these animals.

PROCEDURE

The vitamin A content of the livers was determined for each whole liver separately. The livers were minced, placed in KOH solution and digested within 30 minutes on a water bath. The digest was made up to a volume determined by the liver weight and aliquots used for the analysis. An equal volume of 95% aldehyde-free ethanol and twice the volume of petroleum ether were added, the whole shaken three minutes and centrifuged one minute. Aliquots of the petroleum ether fraction were then removed, the ether evaporated under reduced pressure, and the residue taken up in 0.5 ml chloroform and a drop of acetic anhydride added. The usual antimony trichloride colorimetric procedure was then followed, using the Evelyn photoelectric colorimeter with the 620 filter inserted. This is a modification of the method of Gallup and Hoefer ('46).

An aliquot of the same extract was used for the determination of carotene. The Evelyn photoelectric colorimeter with filter 440 was used and the amount of yellow color determined from a calibration curve obtained with 90% beta- 10% alpha-carotene.

The intestine was washed out several times with normal saline solution, then digested with KOH, and the same procedure employed with the liver was followed. An adaptation of this method was used on the blood. Equal amounts of serum and 5% alcoholic KOH and twice the volume of petroleum ether were shaken for three minutes, then centrifuged for one minute. An aliquot of the ether extract was removed, evaporated and vitamin A and carotene determined as in the liver extract.

Male rats only were used for these experiments. The animals were fed a vitamin A- and E-deficient diet for one week prior to weaning and for two weeks subsequent thereto. The diet fed to the mothers before the animals were weaned consisted of the following, in per cent: vitamin A-free casein 30, olive oil 5, salts 4,[1] yeast 10, cornstarch 51. The basal diet used for all the rats after weaning was the same except that

[1] Hubbell, R. B., L. B. Mendel and A. J. Wakeman, J. Nutrition, *14:* 273 ('37).

the casein was lowered to 22% and the cornstarch correspondingly increased. The oil was first-press imported olive oil and this was used also to dilute the supplements. The carotene was a concentrate derived from vegetable oils.[2] This was filtered before use to eliminate any undissolved material, diluted with olive oil to provide the necessary dosage and its concentration frequently checked against a carotene standard. The animals were dosed by mouth with a calibrated dropper. The water-soluble carotene was prepared fresh at frequent intervals with a dispersing agent, Tween 80 (sorbitan monooleate).[3] This dispersing agent was chosen since it was found that Tweens 20, 21, 40, 60, 61 and 81 produced emulsions with the carotene which was used. The aqueous carotene solutions obtained with these dispersion agents appear to be highly unstable and must be constantly checked for potency. This supplement was fed to the animals by stomach tube since they refused it if given by mouth. In experiments comparing the effect of the two forms of carotene, all the dosing was done by stomach tube.

Pure d-alpha-tocopherol,[4] a distillate of vegetable oils diluted in olive oil, was used. For those animals which were given water-soluble carotene, a similar water-soluble preparation of tocopherol was made and given by stomach tube. Pure vitamin A alcohol was used diluted with olive oil.

The supplements were given three times weekly and in all cases the animals receiving tocopherol were given both carotene and tocopherol at the same time. There was no delayed supplementation.

When the animals were to be sacrificed, they were dosed and killed two to three hours later. The blood was withdrawn from the heart, the livers removed and placed immediately in aqueous 5% KOH, and the intestines were washed out with normal saline, dried on absorbent paper and placed in aqueous 5% KOH as soon as possible.

[2] Obtained from General Biochemicals, Inc.

[3] Obtained from Atlas Powder Company, Wilmington, Delaware.

[4] Supplied by Distillation Products, Inc., Rochester, New York.

The daily dosages of vitamin A and carotene chosen were from 35 to 129 μg of the former and 24 to 174 μg of the latter. Most of the groups were maintained on these levels for 14 days after the preliminary 14 days of depletion, but a few were kept on them for 28 days. The 14-day depletion period was chosen because this was found to be the shortest period in which the livers could be depleted of all vitamin A stores. All the groups, usually of 10 rats, were equalized as to litter origin and sizes in the usual fashion.

RESULTS

In the first series of experiments the rats were killed three hours after being dosed, following the finding of Mattson et al. ('47) that the intestinal vitamin A is highest at this time. Vitamin A was always found in the livers but none in the intestines. Apparently the smaller dosages used in our experiments, 24 to 174 μg of carotene, disappeared much more rapidly from the intestine than the single 900-μg dose used by Mattson et al. This was true also of the vitamin A doses of 38 to 120 μg. No data for intestinal vitamin A are available, therefore, for the series in which oily solutions of vitamin A and carotene were used.

In table 1 the results for the vitamin A series are shown. No consistent differences in either growth, serum or liver vitamin A were ascribable to the 0.5-mg daily dose of α-tocopherol in this series. In only one case, that of the lowest dosage, 35 μg daily for 28 days, was there a significant difference in liver vitamin A in favor of the tocopherol-fed group. The latter group grew more slowly throughout the test and attained a significantly lower final weight than the corresponding non-tocopherol-fed group. The resulting decrease in vitamin A utilization may explain the increased vitamin retention.

At the 75-μg vitamin A daily intake or less the liver stores of vitamin A approximated one-half to two times the daily dose. Apparently an accumulation of the vitamin occurred only when the intake exceeded 75 μg daily. Thus, at 129 μg

for 14 days a store of about 600 μg was found, and at 125 μg for 28 days, a store of about 1,150. These values agree fairly well with the calculated excesses ([129–75] × 14) or 756 and ([120–75] × 28) or 1,260.

There was some indication of a decrease in serum vitamin A levels with increasing liver storage, and also a decrease in rate of growth in the groups which received tocopherol and the larger doses of vitamin A.

The carotene in oil was fed at daily levels of 24 to 174 μg for 14 days and 45 to 135 μg for 28 days. The liver storage of vitamin A approximated one daily dose at only the highest levels. There was no accumulation in any case such as occurred above the 75-μg dosage in the case of vitamin A. Between the total intakes of 1,200 and 2,400 μg of carotene, some advantage in liver storage was seen in the groups receiving the tocopherol. Apparently within the range of 24 to 174 μg carotene daily, no surplus of vitamin A can be built up by

TABLE 1

Effect of varying intakes of vitamin A with and without 0.5 mg α-tocopherol daily on liver vitamin A

(6 to 10 rats in each group)

VITAMIN A INTAKE		α-TOCOPHEROL	PERIOD	GAIN IN WT. IN PERIOD	LIVER				SERUM VITAMIN A
					Wt.	Vitamin A		Carotene total	
Total	Daily					Total	Per gm		
μg	*μg*	*mg*	*days*	*gm*	*gm*	*μg*	*μg*	*μg*	*μg %*
525	38	none	14	39	4.4	20 ± 6	5	5	75 ± 22
525	38	0.5	14	38	5.1	30 ± 7	6	4	61 ± 13
1,050	75	none	14	46	4.9	74 ± 7	15	4	56 ± 18
1,050	75	0.5	14	48	4.6	92 ± 8	20	4	67 ± 10
1,800	129	none	14	75	7.3	607 ± 40	83	4	29 ± 5
1,800	129	0.5	14	70	7.1	573 ± 50	81	4	69 ± 19
980	35	none	28	121	8.6	27 ± 4	3	5	22 ± 0.6
980	35	0.5	28	107	7.8	64 ± 7	8	5	42 ± 15
3,400	120	none	28	128	8.3	1,142 ± 55	138	3	39 ± 12
3,400	120	0.5	28	118	8.1	1,150 ± 109	142	2	44 ± 12

rats of this age and rate of growth. The liver stores found, at least in the lower range, may be considered residues of the carotene administered two to three hours before the animals were sacrificed. In any case the coincidence of similarity in size of daily intake and liver store is striking. The groups fed carotene for 4 weeks instead of two made a slightly better showing, possibly because the growth rate and utilization of vitamin A had been slightly decreased in the latter part of the period. The close relationship between the size of the

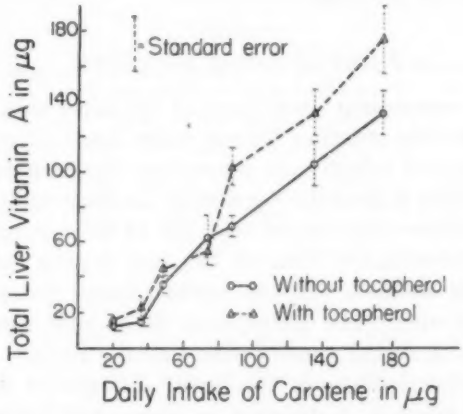

Fig. 1 Effect of 0.5 mg tocopherol daily for 14 days on liver vitamin A of rats given varying daily dosages of carotene in oil.

daily dose of carotene and of the liver store of vitamin A is shown in figure 1. A sharp divergence of the latter values due to the tocopherol at the 87-μg and larger dosages is evident.

As in the case of the vitamin A-fed groups, there was a tendency for the animals which received carotene and tocopherol and which stored greater amounts of vitamin A to gain less weight during the test period than did the groups which received carotene alone. This inverse relationship between growth and liver vitamin A stores has been observed before but the smaller gains by rats fed tocopherol were somewhat unexpected.

Liver carotene

The substance capable of absorbing light using filter 440, although no color appeared, was arbitrarily called carotene. This carotene-like substance, or perhaps carotene itself, was present in a small amount in the livers of all the animals which received vitamin A in about the same minimum amounts (table 1). Larger and more variable quantities, 5 to 37 μg total, were found in the livers of the rats given carotene. The larger intakes of carotene seemed to produce larger but limited deposits in the liver.

Effect of 1.0 mg tocopherol

Another experiment [5] was carried out after this study was completed to test whether 1.0-mg daily doses of α-tocopherol are more or less effective in promoting the transfer of carotene to vitamin A than 0.5-mg doses. An important difference from the earlier experiment was the 24-hour period allowed to elapse between the time of the last dosing and the sacrifice of the animals. In the earlier study the period was three hours only. The kidneys of these rats were assayed for vitamin A in the same fashion as the liver.

In this experiment no significant differences were found among the vitamin A deposits in livers and kidneys of the groups fed, respectively, none, 0.5 or 1.0 mg α-tocopherol, with either 43 or 86 μg carotene daily, for 14 days (table 2). The magnitude of liver deposits was similar to that noted in the first groups (fig. 1) but no larger amounts appeared in the rats fed 0.5 or 1.0 mg tocopherol than in those which received none. The rats fed 87 μg daily and 0.5 mg tocopherol in the first experiment, which had significantly greater liver deposits of vitamin A than did those not given tocopherol, were sacrificed only three hours after the last dosing. The second lot given none, 0.5 or 1.0 mg tocopherol and sacrificed 24 hours after the last dosing, showed no such difference. The utiliza-

[5] We acknowledge gratefully the assistance of Lotte Arnrich and Mary R. C. Nelson in the conduct of this experiment.

tion of reserve vitamin A during the 24-hour period may have masked any differences which might have existed at the three-hour interval. In any case no significant advantage or disadvantage resulted from the use of 1.0-mg instead of 0.5-mg doses of tocopherol. When both liver and kidney reserves of vitamin A were measured, it was still evident that at these levels of intake only a little more than the equivalent of the daily dose can be stored in 14 days.

Swick and Baumann ('52), using a procedure somewhat similar to that of these experiments, found that after 15 days'

TABLE 2

Liver and kidney vitamin A deposits from carotene as affected by α-tocopherol dosage

(9 to 12 rats in each group)

CAROTENE INTAKE		GAIN IN WT. IN 14 DAYS	TOCOPHEROL DAILY	LIVER VITAMIN A		KIDNEY VITAMIN A	TOTAL STORED VITAMIN A
Daily	Total in 14 days			Total	Per gm	Total	
μg	μg	gm	mg	μg	μg	μg	μg
43	600	47	none	32 ± 5	5.1	18 ± 2	50
		55	0.5	38 ± 5	6.2	22 ± 4	60
		49	1.0	39 ± 6	6.7	19 ± 4	58
86	1,200	54	none	83 ± 8	12.7	14 ± 3	97
		56	0.5	91 ± 9	14.8	11 ± 2	102
		60	1.0	96 ± 8	14.4	16 ± 2	112

feeding of 40 μg of β-carotene daily, rat livers and kidneys contained a total of 52 μg vitamin A when no α-tocopherol was given and 33.8 to 59.4 μg when 0.5 mg α-tocopherol was given. Larger doses of α-tocopherol, γ-tocopherol or α-tocopheryl-acetate decreased vitamin A storage in the liver. The stores produced by 37 or 46 μg per day for 14 days in our animals were 15 and 36 without α-tocopherol, and 23 and 44 with 0.5 mg daily. The values are in the same range and in all cases closely related to the daily dosage. Similarly, the liver deposit found by Johnson and Baumann ('48b) when 20 μg β-carotene were given daily for 15 days was 13 μg vitamin A,

the same amount found in our rats fed 24 μg daily for 14 days. When they fed 40 μg daily, these workers found 38.8 μg, comparable with 36 μg in our rats fed 46 μg daily (fig. 1) and 32 μg in our rats fed 43 μg (table 2).

The effect of tocopherol upon utilization of water-dispersed carotene

Since it has been reported by Popper and Volk ('48), Sobel et al. ('48), Kramer et al. ('47) and others, that fat-soluble vitamins are more readily absorbed and utilized if they are rendered water "soluble," an experiment was planned to utilize this form of dispersion. Six groups of animals were used, three of them to receive carotene in oil, and three in the water-dispersed form. Of these, one group of each received carotene alone, another was supplemented with water-dispersed tocopherol by mouth, and the third group received the tocopherol intraperitoneally. The levels were adjusted so that all the groups received essentially the same amount. The results of this experiment substantiated the fact that the carotene in water was more readily absorbed than the carotene in oil, or at least that the liver stores resulting from it were increased. The animals which received water-dispersed carotene alone had the greatest stores of all. Those animals receiving in addition tocopherol by mouth had significantly depressed stores. The administration of tocopherol by the intraperitoneal route apparently was ineffective, since the depressing effect was not noted (table 3). The animals which received carotene in oil responded with small, possibly insignificant, increases in liver stores when tocopherol by either route was given.

The behavior of the first group seemed to indicate either that when carotene can be readily and rapidly absorbed, additional tocopherol becomes a depressant factor, or that tocopherol in the water-dispersed form is less effective than tocopherol in oil. However, the animals which received carotene in oil with tocopherol in the water-dispersed form showed no depressed liver storage. The mechanism of de-

pression of carotene utilization when excessive doses of tocopherol are given may be the same as that seen here with a moderate intake of tocopherol but a rapidly absorbable form of carotene. The rate of absorption of tocopherol may be so much less than that of the water-dispersed carotene that the effective ratios in the intestine may become unfavorable.

The liver stores of all the rats in this series were smaller than those of animals in the earlier series (fig. 1), in which

TABLE 3

Liver storage of vitamin A from carotene fed in oily and aqueous media, with and without 0.5 mg α-tocopherol

(8 to 11 rats in each group)

CAROTENE DISPERSION	TOCOPHEROL DAILY	CAROTENE INTAKE		LIVER			GAIN IN 14 DAYS
		Total	Daily for 14 days	Wt.	Vitamin A		
					Total	Per gm	
	mg	*μg*	*μg*	*gm*	*μg*	*μg*	*gm*
Aqueous	none	1,400	100	4.0	29 ± 3	7.3	33
	0.5 by mouth	1,400	100	5.1	16 ± 2	3.1	42
	0.5 I.P.	1,400	100	5.2	29 ± 4	5.6	44
Oily	none	1,316	94	6.1	16 ± 2	2.6	48
	0.5 by mouth	1,316	94	4.4	20 ± 3	4.6	42
	0.5 I.P.	1,316	94	5.0	19 ± 2	3.8	42

similar levels of carotene were fed. No obvious reason for this was found. Similar variation in response by each series of animals is a common phenomenon. The stomach tube feeding of rats, in our experience, usually results in depressed utilization of the food.

The rate of absorption

The vitamin A of the intestinal wall, free of lumen contents, was determined in the previous experiments three hours after the last carotene-in-oil dosage. No vitamin A was found

in any case after this interval, although in the experience of Mattson et al. ('47), using carotene in oil, the peak of intestinal transformation was reported at the three-hour period. A time study was made, using depleted animals and a single dose of 390 μg of aqueous carotene without tocopherol. Groups of the rats were killed 15 minutes, 30 minutes, one hour, one and one-half hours, two hours, two and one-half hours and three hours after the dosing. Vitamin A was determined in liver and intestine. As is shown in figure 2, vitamin A appeared in the intestine after 30 minutes and in the liver after one and one-half hours. The amount in the intes-

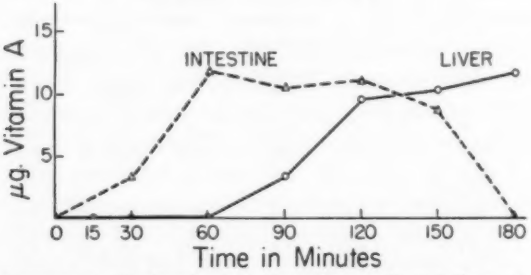

Fig. 2 Appearance of vitamin A in intestinal wall and liver of rats after oral administration of one dose, 390 μg, of aqueous carotene.

tine was increased up to one hour, was maintained up to two and one-half hours and disappeared at three hours. No vitamin A was seen in the liver up to one and one-half hours, after which there was a steady rise up to the end of the experiment at three hours. According to the experience of Shaw and Deuel ('44), carotene continues to disappear from the lumen of the intestinal tract and to appear in the tract wall for 42 hours after the last dosage. These investigators made no vitamin A determinations. The maximum vitamin A accounted for in our experiment was 21 μg at two and one-half hours, with about half this amount seen in the liver at three hours. Obviously the vitamin A was rapidly utilized by these depleted growing animals, or else a large part of the single dose

of carotene was not absorbed up to three hours. Clear evidence is here available that water-dispersed carotene is absorbed and transformed to vitamin A at a much more rapid rate than is the oily dispersion. Mattson et al. ('47) found vitamin A in the liver only after two and three-quarter hours and measurable concentrations of both carotene and vitamin A in the intestine from one and one-half to 5.75 hours. With our water-dispersed carotene, vitamin A appeared in the liver at one hour and in the intestine at one-half hour. Apparently no more vitamin A was present in the intestine after three hours. The absolute quantities present were similar in the two studies, although Mattson et al. gave more than twice as much carotene as was administered in this experiment.

An interfering substance in the intestine

The determination of vitamin A in the intestine of the animals in these experiments had been complicated by the appearance of a pink instead of the expected blue color on the addition of antimony trichloride. This color appeared immediately on addition of the reagent and deepened on standing. It was distinct from the non-specific amber color seen in vitamin A-free extracts. This compound did not appear when vitamin A as such had been given, nor did it appear when any vitamin A was present in the intestine, but only at the 15-minute and three-hour intervals. Since the substance involved must be nonsaponifiable and ether-soluble, bile salts and cholesterol were suspected as being responsible and were tested for, but the former proved to be incapable of producing the pink color reaction. Cholesterol in the amount of 30 mg per milliliter of extract also produced this color, but the petroleum ether extract of the intestine contained only 1% of this amount of cholesterol. However, it was found that this pink color did not appear if any vitamin A was present. It is possible that the interfering substance may be an intermediate in the carotene-to-vitamin A transformation.

SUMMARY

The addition of 0.5 mg α-tocopherol daily to the diet of partially depleted rats receiving 35 to 129 μg vitamin A daily for 14 or 28 days produced no significant change in the liver stores of vitamin A, except in one group on the lowest level of intake for 28 days. Above a 75-μg daily intake of vitamin A, the liver storage was nearly quantitatively cumulative.

Addition of 0.5 mg α-tocopherol supplement daily to the diet of similar rats given 24 to 174 μg of carotene in oil daily for 14 or 28 days produced a significant increase in liver vitamin A when the total carotene intake was from 1,200 to 2,400 μg, or the daily intake from 87 to 174 μg for 14 days. Above and below these levels no effect was found. No cumulative storage of vitamin A was found at any level of dosing.

A consistent but small decrease in growth was seen in the groups which received tocopherol with carotene and profited by it in regard to liver vitamin A. This appeared also in the vitamin A-fed rats, even though the effect on liver vitamin A was negligible.

A later experiment in which 1.0-mg daily doses of α-tocopherol were used as well as 0.5-mg ones indicated that there was no difference in the effectiveness of these levels. In this series the tocopherol produced small and perhaps insignificant increases in liver but not in kidney deposits of vitamin A. The total store of vitamin A in these animals was in all cases only slightly greater than the daily dosage.

The peak of vitamin A content in the intestinal wall was found to occur two hours after carotene in water dispersion had been given, and after three hours no vitamin A was present in the intestine. Vitamin A was also always found in the intestine after two hours but not after three hours in the experiments with carotene in oil. The speed of disappearance may be related to the magnitude of the dose.

An interfering substance which produced a pinkish color instead of the blue of vitamin A with the antimony trichloride reagent appeared in the intestine at 15 minutes and three

hours after the carotene dosing and at no other time. This may be caused by an intermediate in the carotene-to-vitamin-A reaction.

Carotene in aqueous solution (Tween 40) produced larger amounts of liver vitamin A than carotene in the oily medium, but tocopherol was ineffective or depressant under these conditions.

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DIETARY CONSTITUENTS AFFECTING PLASMA AND LIVER CHOLESTEROL IN CHOLESTEROL-FED CHICKS

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The admixture of soybean sterols or of sitosterols with a cholesterol-enriched diet fed to chicks prevents the hypercholesteremia which normally occurs on a high cholesterol diet, and decreases the deposition of cholesterol in the livers of the animals. It has been proposed that the soy sterols may interfere with absorption of cholesterol (Peterson, '51; Peterson et al., '52).

This report is concerned with further studies on relationships among plant sterols and cholesterol, together with some observations on the effects of other dietary additions.

METHODS

The basal diet used in these experiments was the same as that previously reported (Peterson et al., '52), except that it contained 2% of alfalfa meal replacing an equal amount of barley. All other additions to the diet were made at the expense of barley. The basal diet contained by analysis 0.05% cholesterol, 0.02% of other sterols, and 3.00% crude fat (ether extract).

Single-Comb White Leghorn chicks were kept on the laboratory stock diet until they were two or three weeks of age, when they were placed on the experimental diets. In all groups

except in experiment 4, heparinized blood samples were drawn at weekly intervals after the birds had been on the diets for two weeks. Pooled samples of plasma from three chicks were analyzed each week for total cholesterol by the method of Sperry and Webb ('50). Livers taken at autopsy were analyzed as previously described by Peterson ('51). In the tables, only representative analytical values are presented in order to conserve space.

EXPERIMENTAL

Experiment 1 (table 1)

This experiment was conducted to determine if the mode of incorporation of cholesterol, or of cholesterol and soy sterols, into the diet influenced the degree of cholesteremia obtained. The dietary additions are indicated in table 1.

Three methods of incorporating soy sterols,[1] cholesterol and cottonseed oil into the diet were employed: (1) The sterols were stirred into the cottonseed oil and heated just sufficiently to dissolve them. The solution was cooled to a thick paste and blended into the barley. This mixture was then incorporated into the diet with a food mixer. (2) A suspension of the sterols in cottonseed oil was prepared, using a Waring Blendor. This suspension was then mixed into the diet. (3) Cholesterol was dissolved with the aid of heat in half the cottonseed oil and then mixed with half the barley. Soy sterols were similarly dissolved in the other half of the cottonseed oil and mixed with the remainder of the barley. These mixtures were then incorporated into the diet.

Experiment 2 (table 1)

A commercial preparation of soy lecithin [2] was incorporated at the 0.5% level into two diets — one containing 1% cholesterol, the other containing 1% cholesterol plus 1% soy ste-

[1] Ninety-five per cent mixed soy sterols. Distillation Products Industries. Consist principally of sitosterols and stigmasterol.

[2] Alcolec S, American Lecithin Co. Reported to contain 65% phosphatides.

TABLE 1

Effects on plasma cholesterol of several dietary constituents and methods of diet preparation

SUPPLEMENTS	METHOD	PLASMA TOTAL CHOLESTEROL, MG % [1] WEEKS ON DIET			
		2	5	2	5
		Experiment 1 [2]		Experiment 2 [3]	
4% cottonseed oil (basal diet)	.....	107	86	86	79
1% cholesterol	no oil	152	156	..	..
1% cholesterol 1% soy sterols	no oil	98	117	..	..
4% cottonseed oil 1% cholesterol	heat used to dissolve sterols	...	...	610	828
1% cholesterol 1% soy sterols 4% cottonseed oil	heat used to dissolve sterols	148	171	159	194
1% cholesterol 1% soy sterols 4% cottonseed oil	sterols dispersed in oil with Waring Blendor	168	157	..	..
1% cholesterol 1% soy sterols 4% cottonseed oil	cholesterol and soy sterols dissolved separately in oil and added separately to diet	211	252	..	..
1% cholesterol 1% paraffin oil 4% cottonseed oil	mixture prepared by heating	647	782	..	..
4% cottonseed oil 1% cholesterol 0.5% soy lecithin	mixture prepared by heating	...	...	860	1006
4% cottonseed oil 1% cholesterol 1% soy sterols 0.5% soy lecithin	mixture prepared by heating	...	...	148	228

[1] Average of three pooled samples from three chicks each.

[2] Chicks of both sexes on diets at age three weeks.

[3] Male chicks on diets at age three weeks.

rols. The other three diets serving as controls are indicated in table 1. All diets contained 4% cottonseed oil. These various additions were made by dissolving them in cottonseed oil with heating and then mixing the cooled suspension into the

TABLE 2

Effects of varying levels of soy sterols on plasma and liver cholesterol

SUPPLEMENT	(MALE CHICKS ON DIET AT AGE THREE WEEKS)		
	Plasma total cholesterol [1]		Liver total cholesterol [2]
	2 wk. on diet	6 wk. on diet	6 wk. on diet
	mg %	*mg %*	*% dry weight*
4% cottonseed oil	100	83	1.00
4% cottonseed oil 1% cholesterol	752	1226	6.88
4% cottonseed oil 1% cholesterol 0.25% soy sterols	481	787	5.97
4% cottonseed oil 1% cholesterol 0.5% soy sterols	229	349	4.61
4% cottonseed oil 1% cholesterol 1% soy sterols	153	163	2.38
4% cottonseed oil 1% cholesterol 2% soy sterols	100	91	1.37
4% cottonseed oil 1% cholesterol 3% soy sterols	95	108	1.17

[1] Average of three pooled samples from three chicks each.

[2] Average of individual analyses on 4 chicks per group.

diet. Analytical results are shown in table 1. Unfortunately, a technical error invalidated the analyses performed on the livers of these groups.

Experiment 3 (table 2)

The soy sterols may act as inhibitors in an enzyme system involved in cholesterol absorption. It would then be expected

that when a constant level of cholesterol is fed, the plasma cholesterol would decrease and approach a constant level as the concentration of soy sterols in the diet is increased. In this experiment, cholesterol was added at the 1% level in all diets except the basal. Cottonseed oil was included at the 4% level in all diets. Soy sterols were fed at levels of 0.25, 0.5, 1.0, 2.0 and 3.0% of the diets.

Experiment 4 (table 3)

In a previous experiment it was demonstrated that the sitosterol fraction of soybean sterols was also effective in depressing plasma cholesterol on the 1% cholesterol diet (Peterson, '51). In the present experiment mixed sitosterols, ergosterol and stigmasterol were fed with cholesterol. Oil-sterol suspensions were prepared in the Waring Blendor. Because of the limited quantities of pure sterols available, it was necessary to use the younger (two-week-old) chicks and to sacrifice half of them after three weeks and half after 4 weeks on the diet, in order to extend the experiment over a sufficient period of time. Data for the 4th week on the diet are presented in table 3.

Experiment 5 (table 3)

In this experiment the effects of esterifying the hydroxyl groups of both cholesterol and the mixed soy sterols with capric acid were observed. The esters were prepared by the method of Cataline et al. ('44). The cholesteryl caprate was characterized as follows: Turbidity point 81°C., melting point 92.5°C., $[\alpha]_D^{26} = -30.2°$ ($CHCl_3$), as compared with Page and Rudy's ('30) values of 82°C., 93°, and $[\alpha]_D^{20} = -29.8°$, respectively. It contained 0.31% free cholesterol and 1.2% free fatty acid calculated as capric acid. The soy sterol caprates contained 0.36% free sterol and 4.1% free fatty acid calculated as capric acid. Sterol-oil dispersions were prepared in the Waring Blendor. Sterol esters were fed at levels equivalent to 1% free sterol. Soy sterol caprates were fed in the diet with cholesterol. Cholesteryl caprate was fed both with and without the free soy sterols.

TABLE 3

Effects of various sterols and sterol caprates on plasma and liver cholesterol levels

SUPPLEMENT	EXPERIMENT 4 [1]		EXPERIMENT 5 [2]		
	Plasma total cholesterol	Liver total cholesterol	Plasma total cholesterol		Liver total cholesterol
	4 wk. on diet		2 wk. on diet	5 wk. on diet	5 wk. on diet
	mg % (5) [3]	*% dry weight* (5)	*mg %* (9)	*mg %* (9)	*% dry weight* (6)
4% cottonseed oil	73	1.04	83	84	1.79
4% cottonseed oil 1% cholesterol	892	8.67	461	860	12.70
4% cottonseed oil 1% cholesterol 1% soy sterols	142	2.28	175	159	4.95
4% cottonseed oil 1% cholesterol 1% sitosterols	160	2.09	...	...	...
4% cottonseed oil 1% cholesterol 1.5% β-sitosterol	105 [4]	1.82 [4]	...	...	...
4% cottonseed oil 1% cholesterol 1% ergosterol	143	2.74	...	...	...
4% cottonseed oil 1% cholesterol 1% stigmasterol	191	3.56	...	...	...
4% cottonseed oil 1.38% cholesteryl caprate	...	...	137	169	3.97
4% cottonseed oil 1% cholesterol 1.38% soy sterol caprates	...	...	291	521	8.45
4% cottonseed oil 1.38% cholesteryl caprate 1% soy sterols	...	...	118	128	2.33

[1] Mixed sexes on diet at age two weeks.
[2] Mixed sexes on diet at age three weeks.
[3] Figures in parentheses indicate numbers of chicks included in the average values.
[4] Three weeks on diet.

RESULTS AND DISCUSSION

The assumption is made in the following discussion that the levels of cholesteremia and of liver cholesterol in cholesterol-fed chicks are indexes of the degree of cholesterol absorption.

In comparison with chicks fed the basal diet containing cottonseed oil but no added cholesterol, chicks fed cholesterol at the 1% level without extra fat in the diet developed only a moderate cholesteremia (table 1). Stamler and Katz ('51), using a similar type of diet containing 2% cholesterol without added fat, obtained about twice the cholesterol levels obtained here. In both this experiment and that of Stamler and Katz, a much greater degree of cholesteremia occurred when cholesterol was fed with extra fat in the diet, indicating that fat facilitates cholesterol absorption in the chick. This effect of fat on cholesterol absorption may possibly be related to its action in increasing the flow of bile. Siperstein et al. ('52) have shown that in the absence of bile dietary cholesterol is not absorbed by the rat. Paraffin oil evidently had no effect on cholesterol absorption when fed at the level of 1% in the cholesterol-cottonseed oil diet (table 1). It is possible that higher levels of paraffin oil might decrease absorption.

Various methods of adding soy sterols and cholesterol to the diet appeared not to affect greatly the plant sterols' property of inhibiting the rise in plasma cholesterol. When these materials were added to the diet separately, however, slightly higher levels of cholesterol were obtained, possibly because the mixture was not as intimate as in the other methods of preparation (table 1).

Feeding of soy lecithin with cholesterol and cottonseed oil appeared to cause a somewhat greater increase in plasma cholesterol than was obtained on the same diet minus the lecithin (table 1). This is in contrast to the decrease in plasma cholesterol noted in rabbits fed lecithin and cholesterol by Kesten and Silbowitz ('42), although it should be stated that these investigators fed much higher levels of lecithin. It is interesting to note, in this connection, that Stamler et al. ('50) found that choline and inositol increased rather than decreased plasma cholesterol in cholesterol-fed chicks. Addition of soy sterols to the lecithin-cholesterol diet again decreased plasma cholesterol.

Slight deviations in chemical structure from that of the cholesterol molecule, either in the side chain or in the ring structure, appear to confer on the sterol molecule the property of inhibiting cholesterol absorption. Siperstein et al. ('53) have recently shown that dihydrocholesterol, which differs from cholesterol by the presence of two additional hydrogen atoms in the ring structure, also prevents the elevation of plasma cholesterol in chicks fed a high cholesterol diet. Stigmasterol and beta sitosterol, which differ from cholesterol in the structure of the side chain, also have this property (table 3).

These sterols have in common with cholesterol a free hydroxyl group in position 3, as well as other structural similarities. The possibility is suggested that the effect produced is an inhibition of an enzyme system concerned in cholesterol absorption and possibly involving esterification of cholesterol. When the caprate esters of soy sterols were fed with cholesterol, a progressive rise in plasma cholesterol occurred, indicating that these substances require a free hydroxyl group in order to be effective inhibitors (table 3). This rise continued during the entire 5-week feeding period, although it was not as rapid as when cholesterol alone was fed.

When cholesteryl caprate was fed, there was only a very slight increase in plasma cholesterol and the liver cholesterol was about twice that of the birds on the basal diet. This indicates either that esterification of the hydroxyl group interfered with absorption, or that the esterified cholesterol, if absorbed at the same rate as cholesterol, must have been rapidly metabolized. Plasma and liver cholesterol were further depressed when cholesteryl caprate was fed with soy sterols.

When cholesterol was fed with added fat in the diet, hypercholesteremia and excessive deposition of cholesterol in the liver were prevented by additions to the diet of mixed soy sterols, soy sitosterols, β-sitosterol, ergosterol, or stigmasterol (table 3). Cholesterol levels were depressed in proportion to the level of soy sterols in the diet until the ratio of soy sterols to cholesterol was 2:1 or 3:1, when normal plasma

and liver levels of cholesterol were obtained, suggesting again that this phenomenon may be an inhibition of an enzyme system (table 2).

Esterification of cholesterol occurs in the rat during its passage from the intestinal lumen to the lymph (Bollman and Flock, '51; Chaikoff et al., '52). In both normal chickens and hypercholesteremic chickens fed a high cholesterol diet (where the hypercholesteremia represents principally exogenous cholesterol), the esterified cholesterol averages 50 to 70% of the total cholesterol of the plasma (Dauber and Katz, '42; Chaikoff et al., '48). Whether or not this cholesterol is esterified during absorption in the chick has not been demonstrated.

The presence of a cholesterol-esterifying enzyme in the intestinal mucosa of rats has been shown by Swell et al. ('50). These authors have concluded that the major source of this enzyme is the pancreas, since the mucosae of animals described as 95% depancreatized contained very little esterifying activity. Stamler and Katz ('51), however, found that hypercholesteremia was greater than normal in depancreatized chicks fed high cholesterol diets. This latter finding indicates that the pancreas is not essential for cholesterol absorption in the chick and that if an esterifying enzyme is involved, its source in the chick is probably not the pancreas.

The possibility must be considered also that the free sterols form complexes with cholesterol which in some manner interfere with the normal absorption mechanism.

SUMMARY

Studies were made of the effects of a number of dietary additions on plasma and liver cholesterol levels of chicks fed a diet containing 1% cholesterol and 4% cottonseed oil. When the cottonseed oil was omitted from the diet, only a moderate cholesteremia occurred.

Paraffin oil at a 1% level or soy lecithin did not prevent hypercholesteremia, but this condition was prevented when a mixture of soy sterols and soy lecithin was fed.

Mixed soy sterols, mixed sitosterols, beta-sitosterol, stigmasterol and ergosterol were effective inhibitors of the progressive rise of plasma cholesterol and of deposition of cholesterol in the liver.

When cholesterol was fed at a constant level of 1% and soy sterols were fed at varying levels, an optimum effect in depressing both plasma and liver cholesterol levels was obtained when the ratio of soy sterols to cholesterol was 2:1 or 3:1.

Esterification of the soy sterols with capric acid destroyed their ability to prevent an increase in plasma and liver cholesterol. Cholesteryl caprate, in contrast to free cholesterol, did not produce a marked hypercholesteremia, and relatively low liver levels of cholesterol were found when this substance was fed.

A possible mode of action of these plant sterols in decreasing cholesterol absorption is discussed.

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EFFECTS OF VITAMIN B_{12} ON THE UTILIZATION OF CAROTENE AND VITAMIN A BY THE RAT [1]

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At least one missing factor besides vitamin A that is required to promote optimum growth in rats maintained on the U.S.P. vitamin A bioassay diet has been found in liver extract (Cabell and Ellis, '47). This factor is apparently identical with vitamin B_{12} (Hartman et al., '49). It is well established that vitamin A is essential for growth and there seems to exist to some extent an inverse relationship between growth and hepatic vitamin A storage (Johnson and Baumann, '48; High and Day, '51). These observations, coupled with the obscurity of the mechanism of action of vitamin B_{12} in stimulating growth and the supplementing of the U.S.P. vitamin A bioassay diet with either vitamin B_{12} or liver extract (Callison et al., '51), suggested a need for an investigation of the effects of vitamin B_{12} on the storage of vitamin A from carotene and of vitamin A fed to rats. The results of these investigations are herein reported.

MATERIALS AND METHODS

Carotene [2] (90% β- plus 10% α-carotene) was dissolved in peroxide-free ether and added to the required amount of cottonseed oil [3] to give the desired concentration in 0.2 ml

[1] This investigation was supported in part by a research grant, RG-2435, from the National Institutes of Health, Public Health Service. Presented at the 121st meeting of the American Chemical Society in Milwaukee, Wisconsin, March-April, 1952.

[2] Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Wesson.

of the oil. The ether was removed under reduced pressure. The required amount of vitamin A acetate [4] was added directly to cottonseed oil to give the desired concentration in 0.2 ml of the oil for supplementing rations. These solutions were stored at 4°C. under nitrogen in low actinic flasks.

Rats were depleted of vitamins A and B_{12} in essentially the same manner as previously described for the depletion of vitamin A stores (Kelley and Day, '50), except that for comparative purposes some of the rats were fed a vitamin A- and B_{12}-deficient diet containing 2 gm of sulfasuxidine per kilogram during the depletion period and subsequent to supplementation in order to inhibit the possible microbial synthesis of vitamin B_{12}. Both carotene and vitamin A were administered in the same manner as previously indicated (Kelley and Day, '50; High et al., '52). Control animals received, in addition to either carotene or vitamin A, intramuscular injections of 0.2 ml of saline solution every other day, and the experimental groups received every other day intramuscular injections of 0.2 ml of saline solution containing 0.28 μg of vitamin B_{12} as either Cobione,[5] Normocytin,[6] or liver extract.[7] The vitamin B_{12} concentration of the liver extract was determined by the microbiological method of Capps, Hobbs and Fox ('49). An APF supplement [8] was added to the vitamin A- and B_{12}-deficient diet in a concentration of 55 μg per kilogram of diet and fed to the experimental rats with carotene. In these experiments the carotene was administered daily, as previously indicated.

[4] U.S.P. Vitamin A Reference Standard containing 10,000 U.S.P. units of vitamin A per gram, New York, N. Y.

[5] Containing 15 μg of crystalline vitamin B_{12} per milliliter of saline solution, kindly supplied by Merck and Co., Inc., Rahway, N. J., through the courtesy of Dr. D. F. Green, Dr. L. Michaud and Dr. M. A. Schooley.

[6] Vitamin B_{12b} and vitamin B_{12} concentrate of 30 μg per milliliter, kindly supplied by Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y., through the courtesy of Dr. Thomas H. Jukes.

[7] Containing 15 A.P. units per milliliter (see footnote 6).

[8] Containing 12.5 mg of vitamin B_{12} per pound, kindly supplied by Merck and Co., Inc., Rahway, N. J., through the courtesy of Dr. D. F. Green.

The methods of sacrificing the animals and analyzing the tissues for vitamin A were substantially the same as previously indicated (High and Day, '51).

RESULTS

Effects of vitamin B_{12} on the utilization of carotene

Albino rats deficient in both vitamin A and vitamin B_{12} were divided into groups of equal sex and growth rate and supplemented daily for 21 days with 33 μg of carotene dissolved in 0.2 ml of cottonseed oil. Control animals received, in addition to carotene or vitamin A, intramuscular injections of 0.2 ml of saline solution every other day and experimental groups received every other day intramuscular injections of 0.2 ml of saline solution containing 0.28 μg of vitamin B_{12}, as indicated in table 1. In series 2, table 1, 2 gm of sulfasuxidine were added per kilogram of the diet deficient in vitamins A and B_{12} and this ration was fed to rats during the depletion period and subsequent to supplementation in order to inhibit the microbial synthesis of vitamin B_{12}.

Vitamin B_{12} administered as either Cobione, Normocytin, or liver extract increased markedly both growth and vitamin A deposition from carotene fed to rats. Without sulfasuxidine added to the diet, the average increase in growth and hepatic vitamin A deposition was 50 and 37%, respectively. With sulfasuxidine added to the diet the average increase in growth and vitamin A deposition was 62 and 37%, respectively, with an average increase in liver vitamin A deposition of 56%. Under the experimental conditions of this study, if sulfasuxidine reduced the microbial synthesis of vitamin B_{12} in the rat, it seemed logical to expect a greater increase in both growth and vitamin A storage with sulfasuxidine in the diet, as observed in series 2, table 1. No significant differences were found between the effects of vitamin B_{12} from the various sources.

In order to determine whether the intramuscular injection of saline solution had an effect on vitamin A deposition from

TABLE 1

Effects of vitamin B_{12} and APF supplement on the utilization of carotene

SERIES	SUPPLEMENT [1]	NO. AND SEX OF RATS	WEIGHT GAIN	VITAMIN A DEPOSITED	
				Liver	Liver + kidneys
			gm.	μg	μg
1	33 μg carotene + saline solution	8F	36	46.4 ± 2.8	55.9 ± 2.4 [2]
	33 μg carotene + liver extract	8F	52	65.0 ± 5.1	74.9 ± 5.1
	33 μg carotene + Cobione	5F	60	61.1 ± 2.9	67.0 ± 2.9
	33 μg carotene + Normocytin	8F	49	64.6 ± 5.2	74.0 ± 5.4
2	33 μg carotene + saline solution	6(2F)	37	36.6 ± 2.8	50.5 ± 3.2
	33 μg carotene + Cobione	5(2F)	59	57.6 ± 3.3	70.8 ± 3.3
	33 μg carotene + Normocytin	6(1F)	60	56.3 ± 2.3	70.2 ± 2.9
3	33 μg carotene	8(3F)	47	39.7 ± 1.7	52.1 ± 1.4
	33 μg carotene + APF supplement	8(3F)	53	49.9 ± 1.7	62.4 ± 1.4

[1] Liver extract, Cobione and Normocytin, containing 0.28 μg of vitamin B_{12} in 0.2 ml of saline solution, were intramuscularly injected every other day during a 21-day period. Carotene was administered daily for 21 days.

In series 2, 2 gm of sulfasuxidine were added per kilogram of the vitamin A- and B_{12}-deficient diet, and in series 3, an APF supplement was added to the vitamin A- and B_{12}-deficient diet in an amount to supply each animal daily with approximately 55 μg of vitamin B_{12} per kilogram of diet.

[2] The standard error of the mean $= \sqrt{\Sigma(X - \bar{x})^2/n(n-1)}$, where X = individual values, $\bar{x}$ = the group mean, and n = the number of rats per group.

carotene fed, a series was carried out in which one group of animals received carotene only dissolved in cottonseed oil [3] as the supplement, and another group received, in addition to carotene, intramuscular injections of 0.2 ml of saline solution,

[3] See footnote 3, page 203.

as in the experiments with vitamin B_{12}. No significant differences were found between the groups.

Effects of APF supplement on the utilization of carotene

APF was added to the diet deficient in vitamins A and B_{12} in an amount to supply approximately 55 μg of vitamin B_{12} per kilogram of diet. This was sufficient to supply each rat daily with approximately two to three times the vitamin B_{12} administered by injection. No attempt was made in these experiments to control the microbial synthesis of vitamin B_{12}. Control animals received daily supplements for 21 days of 33 μg of carotene in 0.2 ml of cottonseed oil with the deficient diet. Experimental animals received the same quantity of carotene per day with the vitamin A- and B_{12}-deficient diet containing APF. Vitamin B_{12}, in the form of an APF supplement, administered orally significantly increased hepatic vitamin A deposition, by 26% (table 1, series 3).

Effects of vitamin B_{12} on the utilization of vitamin A

Because vitamin B_{12} apparently increased the utilization of carotene for tissue deposition of vitamin A, it was of interest to investigate the effects of the substance on preformed vitamin A. The same general procedure was employed in these experiments as in the experiments with vitamin B_{12} and carotene, except that 13 μg of vitamin A per day were substituted for 33 μg of carotene. In series 3, table 2, 2 gm of sulfasuxidine were added per kilogram of the vitamin A- and B_{12}-deficient diet.

In series 1–2, table 2, vitamin B_{12} was found to increase the growth rate but to be practically without effect on vitamin A deposition, with the exception of series 1, in which a slight increase in vitamin A deposition was observed. With sulfasuxidine, series 3, table 2, the same relative effect, of no

significant difference in vitamin A deposition, existed but an increase in growth rate was observed. The average percentage increase in growth in series 1, 2 and 3 were 15, 41, and 24%, respectively. The slight increase observed in series 1, table 2, may have been due to some unrelated factor or to the lower percentage of increased growth as compared to the controls.

TABLE 2

Effects of vitamin B_{12} on the utilization of vitamin A

SERIES	SUPPLEMENT [1]	NO. AND SEX OF RATS	WEIGHT GAIN	VITAMIN A DEPOSITED	
				Liver	Liver + kidneys
			gm	μg	μg
1	13 μg vitamin A + saline solution	12(7F)	46	32.4 ± 1.7	41.0 ± 1.8
	13 μg vitamin A + Cobione	12(7F)	53	37.5 ± 3.0	46.7 ± 3.0
2	13 μg vitamin A + saline solution	8(7F)	39	34.6 ± 1.8	43.2 ± 1.7
	13 μg vitamin A + liver extract	8(7F)	55	33.7 ± 2.1	43.1 ± 1.9
3	13 μg vitamin A + saline solution	12(2F)	38	39.3 ± 1.4	51.6 ± 1.6
	13 μg vitamin A + Normocytin	12(3F)	47	41.1 ± 1.2	53.4 ± 1.2

[1] Liver extract, Cobione and Normocytin, containing 0.28 μg of vitamin B_{12} in 0.2 ml of saline solution, were intramuscularly injected every other day during a 21-day period. Vitamin A was administered daily for 21 days.

In series 3, 2 gm of sulfasuxidine were added per kilogram of vitamin A- and B_{12}-deficient diet.

DISCUSSION

Although these data suggest that an interrelationship exists between vitamin B_{12} and the metabolism of carotene, sufficient evidence is not available to elucidate the mechanism of action. However, the marked effect of vitamin B_{12} in increasing the utilization of carotene for tissue deposition of vitamin A, as opposed to its comparative ineffectiveness with respect

to the deposition of preformed vitamin A, suggests an effect on the provitamin prior to its conversion to vitamin A. This effect may be concerned with the enzymatic conversion of carotene to vitamin A, the absorption of the provitamin in the alimentary tract, or both.

Evidence is mounting that vitamin B_{12} is involved in methylation reactions which seem to be centered around the metabolism of choline (Schaefer et al., '49, '50; Gillis and Norris, '51; Bennett et al., '51). The data of Popper and Chinn ('42) indicate that choline deficiency impairs the utilization of both carotene and vitamin A for hepatic deposition. On the other hand, Clayton and Baumann ('44) found a normal distribution of vitamin A in tissues of young rats with severe symptoms of choline deficiency. A possible explanation for these seemingly contradictory reports may lie in the difference in the methods employed by these investigators. Popper and Chinn employed as their criterion hepatic vitamin A deposition from both carotene and vitamin A fed daily to rats receiving a diet either adequate or deficient in choline. On the other hand, Clayton and Baumann employed as their principal criterion the depletion of hepatic vitamin A in rats fed diets either adequate or inadequate in choline and either high or low in fat content.

If vitamin B_{12} is involved in the synthesis of choline, or if it serves as a sparer of choline, and if vitamin A storage from carotene fed depends on the choline intake, as suggested by Popper and Chinn ('42), this may suggest an explanation for the observations reported here in regard to carotene. In this connection it is interesting that McCollum and Chow ('50) have reported that an interrelationship exists between vitamin B_{12} and dietary fat. They suggested that vitamin B_{12} is involved in the conversion of carbohydrates to fats.

Since vitamin B_{12} was comparatively ineffective with respect to the deposition of preformed vitamin A, it would appear, on the assumption that the effect of vitamin B_{12} in carotene metabolism is indirect and is concerned principally with its effect on choline metabolism, that choline likewise

would be ineffective with respect to the deposition of preformed vitamin A. A recent report of Guerrant and Thompson ('52) that choline did not increase the utilization of vitamin A for growth in rats maintained on the U.S.P. bioassay diet supplemented with preformed vitamin A is in agreement with this interpretation. It is interesting that the vitamin B_{12}-supplemented rats in our experiments showed a greater growth response than the controls and that each group exhibited approximately the same vitamin A deposition from preformed vitamin A fed. Therefore it is concluded that the greater growth stimulated by feeding vitamin B_{12} is not accomplished at the expense of vitamin A deposition. This does not preclude, however, an interrelationship between vitamin B_{12} and the metabolism and utilization of preformed vitamin A in the rat.

Although further data will be required before conclusive statements can be made relative to the mode of action of vitamin B_{12} on both carotene and vitamin A metabolism, the results of this investigation may have some bearing on the mechanism of action of vitamin B_{12} in stimulating growth. Certainly this interrelationship should be considered. Also studies concerned with carotene and vitamin A utilization should consider the vitamin B_{12} potency of the ration administered. Possibly the concomitant administration of vitamin B_{12} and carotene and perhaps vitamin A may exert beneficial effects on the nutritional state of animals in regard to vitamin A needs.

Investigations in progress are designed to give more information relative to the mode of action of vitamin B_{12} on carotene and vitamin A metabolism.

SUMMARY

Young albino rats deficient in vitamin A and vitamin B_{12} were supplemented daily for 21 days with moderate amounts of either carotene or vitamin A dissolved in cottonseed oil. The intramuscular injection of vitamin B_{12} (Cobione, Normocytin, or liver extract) with or without sulfasuxidine in

the diet increased both growth and tissue deposition of vitamin A from carotene fed. An APF supplement was likewise effective in increasing the utilization of carotene for storage of vitamin A. On the other hand, the vitamin was comparatively ineffective with respect to the utilization of preformed vitamin A for storage, but the growth rates of the vitamin B_{12}-supplemented animals were greater.

The results of this investigation suggest that the concomitant administration of vitamin B_{12} and carotene and perhaps vitamin A may exert beneficial effects on the nutritional state of animals in regard to vitamin A needs.

The significance of these observations is discussed in regard to a possible mode of action of vitamin B_{12} on carotene and vitamin A metabolism.

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THE DISAPPEARANCE OF RADIOACTIVITY FROM THE TISSUES OF RATS OF DIFFERENT AGES AFTER SUBCUTANEOUS ADMINISTRATION OF RADIOVITAMIN B_{12}

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The excretion pattern and the fate of vitamin B_{12} after oral or parenteral administration to different species of animals have been reported in several of our communications (Chow et al., '50a, b, '51). These results and those subsequently obtained by other investigators (Jutton and Parsons, '51; Sokoloff et al., '52; Wolff et al., '51) demonstrate that oral administration of this vitamin brings about excretion primarily in feces and to a smaller extent in urine. On the other hand, parenteral administration in large dosages results in the excretion of a major portion in urine and only a little in feces. Studies (Chow et al., '51) with rats show that healthy adult animals raised on diets containing proteins rich in vitamin B_{12} retain 1 to 2 μg of the injected B_{12} in their tissues. By means of radiovitamin B_{12} tagged with Co^{60}, Rosenblum et al. ('52) and Chow ('52) [1] demonstrated that 4 days after injection as much as 9% of the administered radioactivity was retained in kidneys, 3% in liver, and 1½% in pancreas. From their data, it can be calculated that the total activity in the three organs, which comprised only 4% of the carcass weight, represented 29 % of the total radioactivity in the entire animal. In addition to the high concentration of radioactivity in these three tissues, Harte et al. ('53) found that the radio-

[1] In what follows, vitamin B_{12} tagged with cobalt 60 will be designated B_{12}^*; the crystalline, non-radioactive vitamin will be written as vitamin B_{12}.

activity in liver and pancreas reached a maximum in two to three weeks, and remained at this level for approximately three months. The radioactivity in kidneys, however, came to a peak in two to 4 days and declined rapidly to 10 to 20% of this maximum.

These findings indicate that either the mode of chemical binding of B_{12} in the kidneys differs from that in the other two organs, or that the release of the vitamin from kidneys may be related to the renal function, or both. Since kidney function decreases with advancing age, it is therefore of interest to follow the fate of B_{12} parenterally administered to rats of divergent ages. To this end, different amounts of B_{12}^* were injected into "young" and "old" rats, and the radioactivity in urine samples and in several organs was determined. The results are presented in this communication. In the paper which follows, the effect of age on the urinary excretion of vitamin B_{12} by healthy individuals is reported.

EXPERIMENTAL

Animals

Only male rats were used in this study. The animals in the "young" and the "old" groups were two to three and 14 to 18 months old, respectively. In studies I and II, rats of the McCollum strain raised on the stock diet in our colony were used. In study III, rats were obtained from another laboratory [2] and were raised on a different stock diet. However, in a given experiment, only the "young" and "old" animals from the same source were compared. None of the rats suffered from any obvious deficiencies or acute infections during the period of experimentation.

Radioactive vitamin B_{12}

The radioactive vitamin B_{12}[3] tagged with Co^{60} used in all these experiments had a specific activity of 60 μc per milli-

[2] The rats mentioned were obtained through the kindness of Dr. Mona Oser of the Food Research Laboratories, Inc., Long Island City, New York.

[3] Radioactive vitamin was kindly supplied by Merck and Company on allocation from the Isotope Division of the U.S. Atomic Energy Commission.

gram. Dilutions with unlabeled crystalline vitamin B_{12} were made and are indicated in tables 1 and 2.

TABLE 1

The radioactivity in organs of young and old rats 14 days following injections of radioactive vitamin B_{12}

GROUP [1]	B^*_{12}	SPECIFIC ACTIVITY	BODY WEIGHT	LIVER		KIDNEYS	
				Weight	R [2]	Weight	R [2]
	μg	*microcuries per mg*	*gm*	*gm*		*gm*	
Young (4)	1	12	236	13.2	0.039	2.2	0.042
Old (4)	1		406	12.0	0.040	2.5	0.073
Young (3)	2	6	261	13.8	0.065	2.2	0.102
Old (3)	2		449	13.1	0.060	2.8	0.142
Young (4)	4	1.5	264	14.1	0.160	2.3	0.149
Old (4)	4		484	15.3	0.145	3.0	0.261
Young (3)	8	0.75	290	13.9	0.254	2.3	0.155
Old (3)	8		490	14.6	0.222	3.3	0.408

[1] The number of animals used in each group is given in parentheses.

[2] "R" represents the radioactivity in the organs, expressed as its equivalence in micrograms of administered B^*_{12}.

TABLE 2

The microbiological activity and radioactivity of vitamin B_{12} in 24-hour urine specimens

DOSAGE	SPECIFIC ACTIVITY	OLD RATS		YOUNG RATS	
		Microbiological activity	Radioactivity [1]	Microbiological activity	Radioactivity [1]
μg	*microcuries per mg*	*μg of B_{12}*		*μg of B_{12}*	
1	12	0.198	0.071	0.183	0.040
2	6	0.718	0.411	0.768	0.372
4	1.5	2.23	1.19	2.25	1.17
8	0.75	6.73	1.28	6.30	1.51

[1] Radioactivity expressed as its equivalence in micrograms of administered radioactive B_{12}.

Procedure

All rats were placed in individual metabolism cages and offered our stock diet ad libitum. After three to 4 days of acclimation, 1 ml of a desired amount of B_{12}^* with an adjusted

specific activity was injected subcutaneously. Daily collections of urine were made one day before and two days after administration, but fecal matters were discarded. The total volume for each specimen was made up to 100 ml. The microbial activity was measured according to the procedure of Skeggs et al. ('50). The radioactivity was estimated either by the microbial absorption technique (Davis and Chow, '52) or by superimposing 10-ml specimens directly in planchets. No self absorption corrections were made. After drying, the radioactivity was measured, using a thin window Geiger counter.

RESULTS

In experiment I, young or old rats with average body weights of 261 $\pm$ 9.8 and 457 $\pm$ 13 gm, respectively, were divided into 4 subgroups of three or 4 animals each and were administered a desired dose (1, 2, 4 and 8 μg) of B_{12}* in a single subcutaneous injection. The exact number of animals in each group is given in table 1, in which are also summarized the total radioactivity and the mean weights of livers and kidneys of rats sacrificed two weeks after injection. It can be noted that the radioactivity in the organs of both young and old rats increased with the dosage of injected B_{12}*. At any given level, the mean radioactivity in the livers of the two age groups of animals was essentially the same, but the average radioactivity in the kidneys of the young animals was consistently lower than that in the organs of the old rats. This difference, expressed either as c.p.m. per gram tissue or per total organ, was statistically significant by the analyses of co-variance with $P<0.01$, and indicated that there was greater radioactivity in a given weight of the kidneys of the old rats.

The results of analyses of the urine specimens for microbiological activity, using *L. leichmannii* 4797, and for radioactivity of vitamin B_{12} by measuring absorption by microorganisms are given in table 2. No significant differences in the total excretion of vitamin B_{12} activity in the urine of rats in the "young" and "old" groups, as estimated by either

method, were observed. Simple calculation shows that the amount of B_{12} retained by animals in either age group ranged from 1 to 2 μg per rat, even though the old rats weighed twice as much as the young ones.

The lack of observable differences in the urinary excretion or in the radioactivity of the livers of both groups of animals led us to the belief that the greater radioactivity in the kidneys of the old rats sacrificed two weeks after injection of B_{12}^* was due to a slower rate of disappearance rather than to greater amounts initially retained. This hypothesis is supported by the results of the following two studies. In experiment II, two groups of 4 young and old rats from our own colony were each injected with 4 μg of B_{12}^*. The animals were sacrificed 48 hours later. The radioactivity in livers and kidneys, as well as urine specimens collected 24 hours after injection, was determined. The results demonstrated that the radioactivity in the livers of the two age groups was indistinguishable but that the radioactivity in the kidneys was different, being 0.33 μg $\pm$ 0.04 for the old and 0.28 μg $\pm$ 0.02 for the young rats. This small difference, however, was not significant ($P>0.05$). The data therefore suggest that the ability of the kidneys of the rats of the two age groups to retain B_{12} soon after injection was not very different.

In experiment III, two groups of young and old rats, 14 in each group, were injected subcutaneously with 4 μg of B_{12}^*. Urine collections were made 24 hours before, as well as 24 and 48 hours after, injection. Seven animals in each group were sacrificed two days, and the remaining ones 14 days, after injection. The results of the determination of radioactivity in kidneys and livers by wet combustion, and in urine by direct superimposition on planchets, are given in table 3. The microbiological activity of B_{12} in urine was also determined. No differences in the microbial activity or radioactivity of vitamin B_{12} in urine or the radioactivity in the livers of young or old rats, sacrificed either two or 14 days after injection, were observed. However, the radioactivity in the kidneys of the old animals was again only insignificantly

higher on the second day after injection. The radioactivity in this organ of both groups decreased sharply but at different rates, so that at the end of two weeks the difference became more marked ($P<0.01$). It is also worthwhile to point out that the radioactivity in the livers of both groups was somewhat higher at the end of the second week.

TABLE 3

*The radioactivity of kidneys and liver two and 14 days after injection of B_{12}**

ORGAN	DAYS AFTER INJECTION			
	Two		Fourteen	
	Young	Old	Young	Old
Kidneys B_{12} (mμg)	354 ± 27	405 ± 22	109 ± 10	168 ± 12
Liver B_{12} (mμg)	54.8 ± 4.1	53.0 ± 2.3	71.2 ± 6.4	76.6 ± 5.5
Urine Microbiological activity B_{12} (mμg)	2,000 ± 80	2,100 ± 100		
Radioactivity (c.p.m./24 hr.)	1,130 ± 230	980 ± 133		

DISCUSSION

Studies on the excretion of B_{12}, on its retention by various organs, and on the rates of its disappearance therefrom have yielded data pertinent to tracing the absorption of this vitamin and have also provided background information regarding the treatment of certain diseases with B_{12}. The fate of B_{12} in the body tissues after injection is in a number of ways different from that of some of the vitamins in the B group. Of special interest and importance in relation to the present study is the high concentration of the injected vitamin in organs such as kidneys, pancreas and liver and the length of time for which it is lodged there. The retention of B_{12}* is so tenacious that subsequent injection (Harte et al., '53) of unlabeled B_{12} fails to replace, to any observable extent, the bound

B_{12}*. The extraordinary complexity of the mechanism of B_{12}* retention makes it more difficult and challenging to interpret the results correctly in terms of the physiological role of this vitamin. As an example of this peculiar situation, one may mention the studies on the B_{12} reserves of animals which can be calculated by the isotope dilution technique (Rittenberg and Foster, '40) according to the following equation:

$$\frac{Ru}{Mu} = \frac{Ri}{(Ri + P)},$$

where Ru and Mu are micrograms of B_{12} in urine as measured in terms of radioactivity and microbiological activity, respectively. Ri is micrograms of B_{12}* administered and P is micrograms of B_{12} pre-existing in the "mixable pool."

The data from experiment I are condensed in table 4, from which the "pool" values are calculated. It can be seen that the calculated values are 2 to 4 μg for both the old and the young animals. These values are indicative perhaps only of the order of magnitude, since the equation is only valid if adequate mixing of the injected B_{12}* with the pre-existing vitamins in the tissues took place and if the injected B_{12} was not absorbed by the tissues before complete mixing. It is questionable whether either of these conditions was adequately satisfied. In view of B_{12} binding by some organs, such mixing is limited to the vitamin only in selected tissues. Therefore, the reserve of vitamin B_{12} in animals may be divided into two categories; one, the "fixed reserve" and two, the "dynamic reserve." Our calculated values probably reflect the latter. The magnitude of these values undoubtedly is an important factor in accounting for the failure of correspondence between microbiologically determined vitamin B_{12} activity and that indicated by radioactivity measurement (table 2).

Since B_{12}* in kidneys, liver and pancreas cannot be readily replaced by subsequent injection of B_{12}, these organs must then be considered as sites of the "fixed reserves." Of the three organs, kidneys retained initially the largest amount of radioactivity which, if expressed as c.p.m. per gram or-

gan was approximately 14 times that of liver, generally considered to be a major store of vitamin B_{12}. Furthermore, the radioactivity in the kidneys disappeared more rapidly than that of liver. In addition to these two peculiarities, it is now demonstrated that the amount of radioactivity in the kidneys of the young or old rats was initially the same, but became statistically different two weeks after injection. Such data therefore suggest that the older kidneys were losing the injected B_{12} at a slower rate. This difference may be accounted for on a physiological basis or by the existence of chemical substances capable of binding B_{12}, or both. In discussion of the relationship between age and rates of disappearance there

TABLE 4

B_{12} metabolic pool of young and old rats

DOSAGE	YOUNG		OLD	
	Ru/Mu [1]	Pool	Ru/Mu	Pool
μg		μg		μg
1	0.22	3.6	0.36	1.8
2	0.49	2.1	0.57	1.5
4	0.52	4.2	0.53	3.9

[1] $\frac{Ru}{Mu}$ = ratio of B_{12} determined by radioactivity and by microbiological methods.

is no implication that the observed difference was due solely to the normal process of aging in the animals. Rarely have "old" laboratory animals been entirely free from acute infections or other diseases during their life span, and whether these situations contributed significantly to our observations cannot be certain on the basis of our experiments.

SUMMARY

Vitamin B_{12}* was injected subcutaneously at 4 different levels into "young" and "old" rats. The radioactivity in urine specimens collected 24 and 48 hours after injection, and in kidneys and livers of animals sacrificed two and 14 days after injection, was determined. It was found that at a given level of injection the activity in urine samples or in these organs

was the same in both age groups. The exception was that the kidneys of old rats had greater activity than that of young ones 14 days, but not two days, after injection. These results suggest that the agewise difference in the rate of disappearance of radioactivity from kidneys is associated with the renal metabolism or is related to chemical substances capable of binding B_{12}.

ACKNOWLEDGMENTS

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NUTRITIONAL INTAKE OF CHILDREN

I. CALORIES, CARBOHYDRATE, FAT AND PROTEIN [1]

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TWO FIGURES

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INTRODUCTION

Many cross-sectional and short-time studies of the food intake of children have been reported in the literature, but there are relatively few reports of the dietary intake of a single group of healthy children over a period of years. The determination and evaluation of the food intake of individuals are, at best, a painstaking and tedious project. The most accurate method, involving the weighing of food eaten and laboratory analysis of aliquot samples, is prohibitive because of its requirements in time and personnel unless the number of subjects is small and the duration of the study limited.

A series of nutrition histories, carefully taken and evaluated with appreciation of the errors involved, and with a constant effort to minimize these errors by a variety of techniques, can produce valuable and meaningful data. At the present time, nutrition histories provide the most practical method of determining the nutrient intake of large numbers of individuals for relatively long periods of time.

EXPERIMENTAL

For the past quarter century, the Child Research Council has been studying the growth and development — physical,

[1] This study was aided by a grant from the Nutrition Foundation.

physiological and psychological — of a group of children in the Denver area. These children, who come from "upper middle class" families, are primarily of Northern European extraction. They are enrolled in the study early in the prenatal period and are followed throughout the period of growth and into adulthood.

Nutrition studies were added to the program of the Child Research Council in 1946 and have included all children enrolled in the series since 1944. A history of food intake is obtained for the mother at monthly intervals throughout pregnancy. On the baby, after birth, histories are recorded at monthly intervals for the first 6 months and thereafter at intervals of three months.

The plan of operation of the Child Research Council offers a number of advantages to this type of nutrition study. Since enrollment and participation in the Council are voluntary on the part of the families, they tend to have an appreciation of the aims of the research and a willingness to cooperate. Since no advice is given and no criticism made of the dietary intake, in contrast to the situation in teaching clinics, the nutritionist does not have to contend with the attitude which leads the subject to exaggerate the intake to avoid repeated criticism and correction. There is relatively little limitation in choice of food on the basis of economic restraint; therefore the intake reflects primarily voluntary selection.

The history form used is an adaptation of that developed in the Department of Maternal and Child Health of the Harvard School of Public Health (Burke, '47). It includes: (1) general information on the time of meals, food between meals, appetite, food likes and dislikes, and attention given to the child at meals; (2) a detailed history of the amount and frequency of intake of various foods and food groups; and (3) 4 specific 24-hour intakes, one of which is recorded by the nutritionist during the interview and the other three by the mother on succeeding days. Each interview, requiring about one hour, takes place in the home. Home visits, aside from giving a picture of the home environment, allow the measurement of

serving size when there is doubt, which in turn further impresses the informant with the need for accuracy. Amounts are recorded, wherever possible, in terms of standard household measurements — cup, teaspoon, tablespoon, ounce, and so forth.

The history, with the 4 intakes used as a cross-check, is calculated in terms of specific nutrients from a table compiled from published food value tables (Bowes and Church, '51; U. S. Department of Agriculture, '48, '50). While these figures may not represent accurately the exact nutritive content of the particular foods consumed in this area, the use of the same table for all evaluations tends to make the error constant in all histories.

The data presented in this paper represent 604 histories on 46 children (18 boys and 28 girls) who now range in age from 5 months to 8 years. Only the first 5 years of life are included. Eliminated from the present report are histories on infants who were wholly or partially breast-fed and two single histories on older children who had illnesses of sufficient severity and duration to decrease markedly their food intake for the three-month period. All other histories have been included.

RESULTS AND DISCUSSION

The distribution of intakes shows a skewing which nullifies the use of means and standard deviations (figs. 1 and 2). Instead, these data are presented as the 25th, 50th, and 75th percentiles, thus dividing the group into quartiles, using the highest and lowest values observed to date to show the total range of the data. The percentiles were smoothed visually.

The intake of calories, carbohydrate, fat and protein is presented first as total intake with reference to age but without regard to body size, and secondly as intake per kilogram of body weight (tables 1, 2, 3 and 4). The latter figures were computed separately for each child at each age, using the average of his weights at the beginning and end of the time

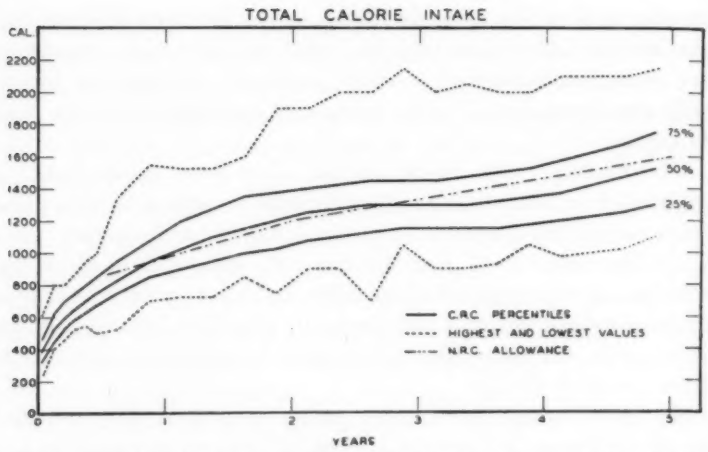

Fig. 1 Total calorie intake of children in the first 5 years of life, showing smoothed 25th, 50th and 75th percentiles and observed minimum and maximum values as contrasted with the recommended dietary allowance.

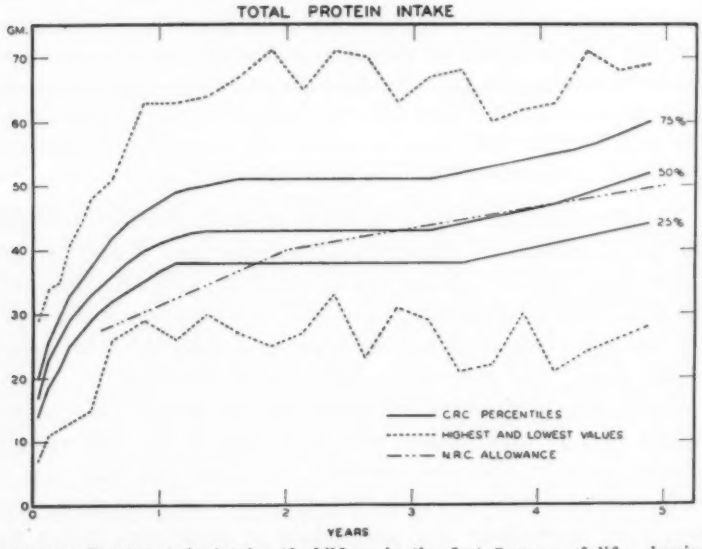

Fig. 2 Total protein intake of children in the first 5 years of life, showing smoothed 25th, 50th and 75th percentiles and observed minimum and maximum values as contrasted with the recommended dietary allowance.

TABLE 1

Caloric intake of children from birth to 5 years of age

AGE		NO. OF CASES	TOTAL CALORIES					CALORIES/KG BODY WEIGHT				
			Percentile					Percentile				
Years	Months		Lowest	25	50	75	Highest	Lowest	25	50	75	Highest
0	0 to 0–1	22	250	300	385	460	615	79	105	118	144	156
0	1 to 0–2	26	400	450	525	625	800	101	122	130	146	165
0	2 to 0–3	26	460	525	600	700	800	89	108	119	127	141
0	3 to 0–4	29	525	585	650	750	875	92	102	110	119	140
0	4 to 0–5	28	550	625	700	800	950	83	98	104	115	136
0	5 to 0–6	29	500	675	750	850	1,000	75	95	102	113	139
0	6 to 0–9	30	525	750	825	950	1,350	65	93	101	111	153
0	9 to 1–0	31	700	850	950	1,075	1,550	74	92	101	112	152
1	0 to 1–3	33	725	900	1,025	1,200	1,525	75	92	103	115	152
1	3 to 1–6	32	725	950	1,100	1,275	1,525	71	92	106	120	139
1	6 to 1–9	31	850	1,000	1,150	1,350	1,600	80	92	106	122	140
1	9 to 2–0	31	750	1,025	1,200	1,375	1,900	63	91	106	122	154
2	0 to 2–3	27	900	1,075	1,250	1,400	1,900	81	90	105	120	149
2	3 to 2–6	27	900	1,100	1,275	1,425	2,000	76	89	102	116	151
2	6 to 2–9	27	700	1,125	1,300	1,450	2,000	53	87	99	113	144
2	9 to 3–0	25	1,050	1,150	1,300	1,450	2,150	69	84	95	111	148
3	0 to 3–3	21	900	1,150	1,300	1,450	2,000	59	82	92	109	131
3	3 to 3–6	21	900	1,150	1,300	1,475	2,050	68	79	90	108	134
3	6 to 3–9	21	925	1,150	1,325	1,500	2,000	59	76	88	107	128
3	9 to 4–0	20	1,050	1,175	1,350	1,525	2,000	62	73	86	106	126
4	0 to 4–3	18	975	1,200	1,375	1,575	2,100	62	71	85	105	126
4	3 to 4–6	17	1,000	1,225	1,425	1,625	2,100	63	70	85	105	126
4	6 to 4–9	16	1,025	1,250	1,475	1,675	2,100	59	70	85	105	116
4	9 to 5–0	16	1,100	1,300	1,525	1,750	2,150	58	70	85	105	122

TABLE 2

Carbohydrate intake of children from birth to 5 years of age

AGE		NO. OF CASES	TOTAL CARBOHYDRATE, GM					CARBOHYDRATE, GM/KG BODY WEIGHT				
			Percentile					Percentile				
Years	Months		Lowest	25	50	75	Highest	Lowest	25	50	75	Highest
0	0 to 0–1	22	21	26	38	56	68	6.1	8.6	12.1	13.9	18.6
0	1 to 0–2	26	31	40	54	66	79	8.2	11.4	13.2	15.5	19.4
0	2 to 0–3	26	36	49	62	74	96	7.7	10.5	12.6	14.2	19.1
0	3 to 0–4	29	42	57	70	82	108	7.1	10.0	12.1	13.8	18.9
0	4 to 0–5	28	41	65	77	90	130	6.7	9.8	11.6	13.5	19.6
0	5 to 0–6	29	52	70	84	98	143	6.3	9.6	11.2	13.3	17.9
0	6 to 0–9	30	61	80	95	110	168	7.2	9.6	11.3	13.2	19.0
0	9 to 1–0	31	56	92	108	128	219	6.4	10.2	11.8	13.6	22.2
1	0 to 1–3	33	71	100	118	142	183	7.4	10.2	12.0	14.2	20.6
1	3 to 1–6	32	84	107	127	153	193	8.4	10.2	12.0	14.4	18.8
1	6 to 1–9	31	81	112	133	162	228	7.7	10.2	12.0	14.4	19.4
1	9 to 2–0	31	55	116	138	167	262	4.6	10.0	12.0	14.4	21.5
2	0 to 2–3	27	89	119	142	170	248	7.1	9.7	11.9	14.2	21.0
2	3 to 2–6	27	91	121	144	172	318	7.5	9.4	11.7	14.0	24.1
2	6 to 2–9	27	60	122	146	174	300	4.5	9.1	11.4	13.7	21.6
2	9 to 3–0	25	102	123	147	176	286	7.1	8.9	11.1	13.4	19.6
3	0 to 3–3	21	91	125	148	178	226	6.0	8.8	10.8	13.1	16.2
3	3 to 3–6	21	105	128	150	182	268	6.5	8.6	10.5	12.9	17.5
3	6 to 3–9	21	102	131	152	186	272	6.4	8.5	10.3	12.7	17.4
3	9 to 4–0	20	98	135	156	192	272	5.7	8.4	10.2	12.5	16.4
4	0 to 4–3	18	103	139	160	198	260	6.0	8.3	10.1	12.3	15.6
4	3 to 4–6	17	127	143	164	206	260	7.3	8.2	10.0	12.2	15.6
4	6 to 4–9	16	113	148	169	214	262	6.1	8.1	10.0	12.1	15.3
4	9 to 5–0	16	110	153	174	222	300	5.8	8.0	10.0	12.0	17.0

TABLE 3

Fat intake of children from birth to 5 years of age

AGE		NO. OF CASES	TOTAL FAT, GM					FAT, GM/KG BODY WEIGHT				
			Percentile					Percentile				
Years	Months		Lowest	25	50	75	Highest	Lowest	25	50	75	Highest
0	0 to 0–1	22	11	16	18	22	30	3.3	4.9	5.5	6.9	7.7
0	1 to 0–2	26	17	21	25	29	38	4.5	5.3	6.3	7.2	7.5
0	2 to 0–3	26	20	23	27	32	44	3.7	4.8	5.6	6.2	6.8
0	3 to 0–4	29	20	25	30	34	49	3.2	4.2	5.0	5.7	6.7
0	4 to 0–5	28	1	27	31	35	53	0.1	4.0	4.6	5.3	5.8
0	5 to 0–6	29	1	28	32	37	53	0.2	3.8	4.5	5.1	5.3
0	6 to 0–9	30	8	30	34	39	52	0.8	3.6	4.3	4.9	6.0
0	9 to 1–0	31	10	33	38	44	77	0.9	3.6	4.3	4.9	7.2
1	0 to 1–3	33	24	36	43	49	72	2.5	3.7	4.3	5.0	5.9
1	3 to 1–6	32	23	40	47	54	64	2.1	3.7	4.4	5.0	6.9
1	6 to 1–9	31	28	43	50	57	71	2.4	3.7	4.4	5.0	6.4
1	9 to 2–0	31	27	45	52	59	77	2.2	3.7	4.4	5.0	6.3
2	0 to 2–3	27	29	46	54	61	78	2.3	3.7	4.4	5.0	6.5
2	3 to 2–6	27	42	47	55	62	79	3.0	3.7	4.3	5.0	6.4
2	6 to 2–9	27	34	48	56	64	89	2.6	3.7	4.3	5.0	6.2
2	9 to 3–0	25	39	48	56	65	89	2.8	3.6	4.2	4.9	6.3
3	0 to 3–3	21	40	48	56	66	90	2.6	3.5	4.1	4.8	5.9
3	3 to 3–6	21	30	48	56	67	78	2.4	3.4	4.0	4.7	5.7
3	6 to 3–9	21	29	49	57	68	79	1.8	3.3	3.9	4.6	5.5
3	9 to 4–0	20	39	50	58	69	84	2.4	3.2	3.8	4.6	5.4
4	0 to 4–3	18	42	51	59	70	90	2.4	3.1	3.8	4.6	5.4
4	3 to 4–6	17	41	53	61	72	90	2.6	3.0	3.8	4.6	5.7
4	6 to 4–9	16	42	55	63	74	92	2.4	3.0	3.8	4.6	5.0
4	9 to 5–0	16	45	57	66	77	98	2.4	3.0	3.8	4.6	5.6

TABLE 4

Protein intake of children from birth to 5 years of age

AGE		NO. OF CASES	TOTAL PROTEIN, GM					PROTEIN, GM/KG BODY WEIGHT				
			Percentile					Percentile				
Years	Months		Lowest	25	50	75	Highest	Lowest	25	50	75	Highest
0	0 to 0–1	22	7	14	17	20	29	2.6	4.3	5.1	6.2	7.4
0	1 to 0–2	26	11	19	23	26	34	2.9	5.0	5.6	6.4	6.8
0	2 to 0–3	26	12	21	26	29	35	2.7	4.4	5.1	5.5	6.0
0	3 to 0–4	29	13	25	29	33	41	2.6	4.1	4.7	5.1	6.3
0	4 to 0–5	28	14	27	31	35	44	2.4	3.9	4.5	4.9	5.7
0	5 to 0–6	29	15	29	33	37	48	2.3	3.9	4.4	4.9	6.4
0	6 to 0–9	30	26	32	36	42	51	3.3	3.9	4.4	4.9	5.8
0	9 to 1–0	31	29	35	40	46	63	2.9	3.8	4.4	5.0	5.9
1	0 to 1–3	33	26	38	42	49	63	2.9	3.7	4.3	4.9	6.2
1	3 to 1–6	32	30	38	43	50	64	2.9	3.6	4.2	4.8	6.4
1	6 to 1–9	31	27	38	43	51	67	2.5	3.4	4.0	4.6	6.1
1	9 to 2–0	31	25	38	43	51	71	2.0	3.2	3.8	4.4	5.8
2	0 to 2–3	27	27	38	43	51	65	2.3	3.1	3.6	4.2	5.6
2	3 to 2–6	27	33	38	43	51	71	2.6	3.0	3.4	4.0	5.4
2	6 to 2–9	27	23	38	43	51	70	1.9	2.9	3.3	3.8	5.1
2	9 to 3–0	25	31	38	43	51	63	2.4	2.8	3.2	3.7	4.6
3	0 to 3–3	21	29	38	43	51	67	2.2	2.7	3.1	3.6	5.3
3	3 to 3–6	21	21	38	44	52	68	1.7	2.6	3.0	3.5	5.3
3	6 to 3–9	21	22	39	45	53	60	1.4	2.5	2.9	3.5	4.2
3	9 to 4–0	20	30	40	46	54	62	1.8	2.5	2.8	3.4	4.2
4	0 to 4–3	18	21	41	47	55	63	1.2	2.4	2.8	3.4	4.3
4	3 to 4–6	17	24	42	49	56	71	1.4	2.4	2.8	3.4	4.7
4	6 to 4–9	16	26	43	50	58	68	1.8	2.4	2.8	3.4	4.4
4	9 to 5–0	16	28	44	52	60	69	1.5	2.4	2.8	3.4	4.2

interval involved; these weights were taken at the child's regular examinations by the Child Research Council staff.

Of particular interest are the comparative rates of increase in the total intake of the 4 nutrients and the clarity with which they reflect the eating problems so commonly encountered with the preschool child. During the first 15 to 18 months the rise in intake of protein is most marked, that of carbohydrate and fat less marked. There follows a stationary level of protein intake, while carbohydrate and fat intakes continue a slow but steady increase, until shortly after three years, when protein again reveals an ascending curve. Calorie intake is, of course, a composite of these three nutrients and shows only a slight increase between 18 months and three years.

The preschool period has long been accepted as an interval in which the child presents a number of problems in eating. There is a lag in appetite, an indifference to food, and an outright refusal of some foods. It has been our experience with this group of children that the most marked decrease is in the consumption of milk and vegetables. The age of onset of poor appetite varies from one child to another, being most often between 9 and 18 months but occasionally later, and the duration varies from a few months to more than two years. To date none of the children in our series has passed through this post-infancy period without a noticeable change in his reaction to food. In future publications we will deal more completely with this phase of food intake in children.

There is a sex difference in intake, which will be reported in detail when more data have been accumulated. However, median calorie intakes of boys and of girls were computed for purposes of comparison with the results of a similar study of children from families of lower economic status in Boston (Beal, Burke and Stuart, '45) in which only the 24-hour intakes were used. After the age of two years, the median of the Child Research Council boys is almost identical with the mean for boys in Boston. By contrast, the median for Child Research Council girls is consistently lower than the mean of

the Boston girls, and the sex difference is more pronounced in the Child Research Council data.

When the protein values were subdivided to show the contribution of various dietary sources to the total intake, it was found that in this series milk contributes almost all of the protein for the first three months, with plant sources beginning in the third month and meat and egg protein in the 6th month. Plant sources increase steadily between three and 15 months of age, contributing 20 to 25% of the total protein intake from 15 months to 5 years of age. Both animal and plant sources show no absolute increase between 15 months and three years of age; then both resume their ascending curve. In a further breakdown of the animal protein, the data show that after an initial rise to a median of nearly 27 gm daily by 6 months, milk protein decreases to its lowest level of 20 gm by three years, rising again to 26 gm by 5 years. Meat protein increases relatively rapidly from 6 months to 18 months to a level of nearly 8 gm daily, then shows no further increase until after 4 years. Egg protein increases between 6 months and 21 months and then shows a slow decline which is still apparent at 5 years.

There is a greater spread of observed intakes above the 75th percentile than below the 25th percentile for all 4 nutrients; this is particularly true with respect to carbohydrate. However, the lowest values for protein during the first year were far below the 25th percentile because two infants were given formulas simulating breast milk and therefore much lower in protein than any other formulas prescribed for this group. The lowest observed fat intakes were especially low in the first year because skim milk formulas were recommended by their pediatricians for two boys, one because of allergy and the other for weight control. With these exceptions, the tendency of these data to be skewed in the direction of high intakes is quite consistent.

A comparison of the median calorie intake of this group of children with the recommended dietary allowances of the Food and Nutrition Board of the National Research Council ('48)

shows relatively close agreement (fig. 1). At no time is the allowance above the 75th percentile or below the 25th percentile. There is, however, somewhat more discrepancy in the case of protein (fig. 2). In the first two years, the recommended allowance is between the minimum and 25th percentile of the Child Research Council series; after two years of age, the recommended allowance is close to the median.

The median calorie intake per kilogram of body weight decreases from a high point of 130 calories in the second month to 85 calories at 5 years. There is a slight increase in the 50th and 75th percentiles during the second year, at a time when the total calorie intake is increasing but when growth increments, both in height and weight, are at their lowest level for this 5-year span (Boyd, '52). This slight increase in intake per kilogram of body weight is observed also in carbohydrate and fat but not in protein. Protein intake per kilogram of body weight decreases in a somewhat more consistent manner from a high of 5.6 gm at two months to 2.8 gm at 5 years.

Individual children show a variety of patterns of intake of these nutrients. Some children maintain a fairly constant level in relation to the rest of the group, such as, for example, a boy whose intake of protein, except for a variable period between the ages of two and three years, has been close to the 75th percentile, in contrast to a girl whose protein intake has always been below the 25th percentile. Other children may have a marked variation in intake from time to time. The individual patterns will be presented in greater detail in future publications.

SUMMARY

Data have been presented from 604 nutrition histories on 46 children in the first 5 years of life. Calorie, carbohydrate, fat and protein intakes, referred to age and to body weight, have been computed in terms of quartiles and maximum and minimum intakes.

Total intakes of calories, carbohydrate and fat increase throughout the period from birth to 5 years. Intake of protein, however, reaches a plateau between 18 months and three

years, reflecting the capricious eating habits of the preschool child. The median calorie intake is close to the recommended dietary allowance of the National Research Council; the median protein intake is above the recommended dietary allowance in the first two years and thereafter is similar to the allowance.

Intake of protein per kilogram of body weight shows a consistent decrease in this age period. The descending curve of intake of calories, carbohydrate and fat per kilogram of body weight is interrupted by a temporary rise in the second year.

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IMPROVING THE NUTRITIVE VALUE OF CEREAL GRAINS

I. IMPROVEMENT IN THE EFFICIENCY OF THE PROTEINS IN MILLED WHEAT FLOUR WITH LYSINE, VALINE, THREONINE AND AN EXTRACT FROM CONDENSED FISH SOLUBLES [1]

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It has been known for many years that the proteins of wheat can be improved by the addition of lysine (Mitchell and Smuts, '32). The recent report that the proteins in whole wheat can be further improved by valine, and considerably more by the addition of threonine (Sure, '52a), stimulated supplementation of the proteins in vitamin-enriched wheat flour with lysine, valine, threonine, and also with an extract from condensed fish solubles, since the latter improved the proteins of a processed whole wheat cereal breakfast food (Sure, '52b).

EXPERIMENTAL

This study was carried out on the Wistar strain albino rat and in all of the groups the sexes were equally divided. The animals were about 30 days old when started on the experiments and they weighed 50 to 54 gm each. The composition of the rations is given in table 1.

[1] Aided by a grant from the Williams-Waterman Fund of The Research Corporation. Research paper 1078 Journal Series, University of Arkansas. Published with the approval of the Director of the Arkansas Agricultural Experiment Station.

A preliminary report of this paper was presented before the Pacific Slope Biochemistry Symposium at The University of California, Berkeley, October 11th, 1952, in connection with the dedication of the Biochemistry and Virus Building.

The following components of the vitamin B complex were administered separately from the ration to each animal 6 times weekly, and a double allowance was given on Saturdays: *during growth:* 25 μg each of thiamine, riboflavin, pyridoxine, and niacin; 150 μg calcium pantothenate; 3 mg *p*-aminoben-

TABLE 1

Composition of rations (%)

CONSTITUENTS	RATION											
	A	B	C	D	E	F	G	H	I	J	K	L
Milled wheat flour	86.4	86.4	86.4	86.4	86.4	84.8	86.4	86.4	85.9	84.3	..	..
Dried whole egg	..	..	..	..	..	..	..	..	..	..	23.5	..
Dried non-fat milk solids	..	..	..	..	..	..	..	..	..	..	..	28.7
Cellu flour	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
L-lysine	..	0.4	..	0.4	0.4	0.4	0.4	0.4	0.4	0.4	..	..
DL-threonine	..	..	0.2	0.2	0.2	0.2	..	0.5	0.5	0.5	..	..
DL-valine	..	..	..	..	0.7	0.7	0.7	0.7	0.7	0.7	..	..
DL-tryptophan	..	..	..	..	..	..	..	..	0.5	0.5	..	..
Sure's salts 1 [1]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Vegetable shortening	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	8.0	8.0
Cod liver oil	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Wheat germ oil	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Fish soluble extract [2]	..	..	..	..	..	1.6	..	..	..	1.6	..	..
Cerelose	1.6	1.2	1.4	1.0	0.3	0.3	0.5	..	..	..	59.5	54.3

[1] Sure, B. The existence of a new dietary factor essential for lactation. J. Nutrition, *22:* 499, 1941.

[2] Equivalent to 5 gm condensed fish solubles.

zoic acid; 1 mg inositol, and 6 mg choline chloride; *during reproduction:* 50 μg each of thiamine, riboflavin pyridoxine, and niacin; 300 μg calcium pantothenate, 6 mg *p*-aminobenzoic acid, 3 mg inositol, 9 mg choline chloride, 50 μg folic acid, 5 μg biotin, and 0.5 μg vitamin B_{12}; *during lactation:* 100 μg each of thiamine, riboflavin, pyridoxine, and niacin; 600 μg calcium pantothenate, 12 mg *p*-aminobenzoic acid, 3 mg inositol, 12 mg choline chloride, 80 μg folic acid, 10 μg biotin, and 1.0 μg vitamin B_{12}.

The fat-soluble vitamins A, D, and E were furnished by the cod liver oil and wheat germ oil in the rations. However, during reproduction and lactation periods the following sup-

plements were administered separately from the ration: three drops of halibut liver oil [2] given three times weekly per animal, and three drops of mixed tocopherol concentrate [3] given once weekly per animal. In addition, 200 μg vitamin K were administered 6 times weekly, and a double dose on Saturdays, to the pregnant and lactating females.

Supplementation of wheat flour with lysine, threonine and valine

Growth and protein efficiency ratio. The results of this investigation are summarized in tables 2, 3, and 4. There were 12 animals in each group represented in table 2. It will be noted from this table that the addition of 0.4% L-lysine in the ration was accompanied by 190% increased growth and an increase in the protein efficiency ratio (PER) of 201%. The addition of 0.2% DL-threonine produced a slight inhibitory effect on growth. However, the supplementation of the basal ration with 0.2% of this amino acid in the presence of L-lysine was accompanied by 306% increased growth and a 236% increase in the PER. The greater increase in body weight following the addition of DL-threonine to ration D was due largely to increased food consumption; however, there was also a 35% increase in PER. The further addition of 0.7% DL-valine to ration E produced 450% increased growth and an appreciable increase in PER, the increase in body weight being again chiefly due to increased food intake. The further supplementation of the basal ration with an extract from condensed fish solubles (ration F) resulted in 526% increased growth, due largely to greater protein efficiency, the PER being 3.11 in spite of a slightly lower total food and, therefore, also of protein, intake. The extract from condensed fish solubles was prepared by precipitating the raw product with 5 volumes of 95% ethyl alcohol. The liquid portion was

[2] The halibut liver oil contained 60,000 U.S.P. vitamin A units, and 850 U.S.P. vitamin D units, per gram, and 1,250 units of vitamin A and 18 units of vitamin D per drop.

[3] The tocopherol concentrate contained 340 mg mixed tocopherols per gram.

TABLE 2

Influence of lysine, threonine, valine and an extract from condensed fish solubles on efficiency of proteins in milled wheat flour [1]
(Experimental period — 6 weeks. Average results per animal.)

RATION	TYPE OF RATION	GAINS IN BODY WEIGHT		TOTAL FOOD INTAKE	PROTEIN INTAKE	PROTEIN EFFICIENCY		NITROGEN INTAKE	GAINS IN BODY WEIGHT PER GM OF NITROGEN INTAKE	INCREASE
		Weight	Increase			Ratio [2]	Increase			
		gm	%	*gm*	*gm*		%	*gm*	*gm*	%
A	Milled wheat	20.6	..	327.3	29.7	0.69 ± 0.05 [3]	..	5.211	3.95	..
B	Milled wheat + 0.4% L-lysine	59.7	190	316.2	28.7	2.08 ± 0.18	201	5.228	11.42	189
C	Milled wheat + 0.2% DL-threonine	18.2	— 13	343.1	31.1	0.59 ± 0.05	— 14	5.496	3.33	— 15
D	Milled wheat + 0.4% L-lysine + 0.2% DL-threonine	83.6	306	397.6	36.1	2.32 ± 0.07	236	6.625	12.62	212
E	Milled wheat + 0.4 L-lysine + 0.2% DL-threonine + 0.7% DL-valine	110.6	450	470.6	42.7	2.59 ± 0.09	275	8.017	13.70	247
F	Milled wheat + 0.4% L-lysine + 0.2% DL-threonine + 0.7% DL-valine + extract from condensed fish-solubles	131.0	526	470.0	41.8	3.11 ± 0.09	351	8.780	14.92	277
K	Dried eggs	120.6	..	429.0	42.9	2.81 ± 0.07	..	6.864	17.55	..
L	Dried non-fat milk solids	124.5	..	531.0	53.1	2.35 ± 0.06	..	8.496	14.64	..

[1] All rations contained 9.07% protein, with the exception of ration F, which contained 8.9%, and rations K and L which had 10%.
[2] Expressed as gains of body weight per gram of protein intake.
[3] Standard deviation of the means.

used in amounts equivalent to 5% of the crude product in the ration. It was poured on the cerelose and contributed 0.196% foreign nitrogen to the ration. The supplementary effect of this extract is probably due to foreign nitrogen but may be also partly due to the effect of an unidentified growth factor (King and Hauge, '49).

TABLE 3

Influence of lysine, valine and threonine on economy of food utilization
(7 gm fed daily to each animal. Experimental period — 6 weeks. Average results per animal.)

RATION	TYPE OF RATION	GAINS IN BODY WEIGHT		GAINS IN BODY WEIGHT PER 100 GM OF FOOD INTAKE	INCREASE
		Weight	Increase		
		gm	%	*gm*	%
A	Milled wheat	21.3	..	7.3	..
B	Milled wheat + 0.4% L-lysine	51.7	145	17.6	141
G	Milled wheat + 0.4% L-lysine + 0.7% DL-valine	52.3	146	17.8	144
H	Milled wheat + 0.4% L-lysine + 0.7% DL-valine + 0.5% DL-threonine	65.5	208	22.3	206
	Same animals as above on ad libitum feeding, subsequent 6 weeks[1]				
A	Milled wheat	8.7	..	3.5	..
B	Milled wheat + 0.4% L-lysine	48.7	460	11.7	234
G	Milled wheat + 0.4% L-lysine + 0.7% DL-valine	64.0	636	14.7	320
H	Milled wheat + 0.4% L-lysine + 0.7% DL-valine + 0.5% DL-threonine	91.0	946	18.6	431

[1] The food consumption per animal during this 6-week period was as follows: ration A, 246 gm; ration B, 416 gm; Ration G, 437 gm; and ration H, 490 gm.

It must be realized that in supplementing the proteins of milled wheat flour with the micronutrients shown in table 1, significant amounts of additional nitrogen were contributed by the amino acids and the extract from condensed fish solubles. Therefore, in addition to data on protein efficiency ratios, figures are presented on gains in body weight per gram of nitrogen intake. In calculating the nitrogen contributions of the amino acids in the rations it was taken into consideration that only the D-threonine (West and Carter, '37) and the L-valine (White, Fones and Sober, '52) are uti-

TABLE 4

Influence of vitamin B_{12} on growth

(12 males and 12 females on each ration. Experimental period — 12 weeks. Average results per animal.)

TYPE OF RATION	GAINS IN BODY WEIGHT		FOOD CONSUMPTION		GAINS IN BODY WEIGHT PER 100 GM OF FOOD INTAKE	
	Males	Females	Males	Females	Males	Females
	gm		*gm*		*gm*	
Milled wheat flour + 0.4% L-lysine	111	110	819	918	13.56	11.95
Milled wheat flour + 0.4% L-lysine + 0.1 μg vitamin B_{12} per animal per day	133	116	918	992	14.53	11.61

lized by the rat; consequently, only one-half of the nitrogen of DL-threonine and one-half of the nitrogen of DL-valine were added to the total nitrogen furnished by the proteins in the rations. On the basis of similar total nitrogen intake, ration F, which is the basal diet supplemented with lysine, threonine, valine, and an extract from fish solubles, approximately equalled the biological value, from the standpoint of growth, of a ration containing 10% proteins derived from dried nonfat milk solids (ration L), but did not equal the efficiency of nitrogen utilization of a ration supplying 10% proteins derived from dried whole eggs (ration K, table 2).

Influence of vitamin B_{12} supplementation of ration B containing lysine. From table 4 it is apparent that 0.1 μg of vitamin B_{12} per animal per day as a supplement to ration B produced an appreciable response in growth, during an experimental period of 12 weeks, in males (18.8%), due largely to increased food consumption, but a negligible increase in growth in females (5.7%).

Reproduction and lactation. Only the group of animals on rations E and F were bred, when they were about 4 months old and when the females each weighed 160 gm or over. The reproduction record was very satisfactory. After being bred for 118 days, 12 females on ration E had 12 litters of 90 young, and on ration F 12 females had 12 litters of 94 young.

During the lactation period, the threonine content was increased from 0.2% to 0.5% in the rations, which were fortified with 0.5% DL-tryptophan. The addition of this essential amino acid was made since Block and Mitchell ('46–'47) reported that the proteins in wheat have a 20% deviation from the corresponding values of whole egg proteins, and since it was anticipated that at the low level of 9.07% protein, the rations, while satisfactory for growth and reproduction, might be inadequate for rearing of the young. The addition of tryptophan was made after a number of litters had died during the first few days of lactation. The final results with respect to lactation on rations I and J were as follows: On ration I, out of 62 young given to rear, 48.4% were weaned, but only by prolonging the lactation period from 22 to 25 days, the "normal" on our stock diet, to 40.8 days. On ration J, out of 60 young given to rear, 48.3% were weaned in 33.4 days, indicating that the extract from fish solubles had the effect of reducing the lactation period by about one week. The inability to secure optimum success in lactation on the basal diet, containing 9.07% proteins in white flour, supplemented with lysine, valine, threonine and an extract from fish solubles, may have been due to the need for additional essential amino acids, such as leucine, isoleucine and arginine (Block and Mitchell, '46–'47), or to an imbalance of the B vitamins, since

we do not know as yet the optimum requirements for the various components of the vitamin B complex for lactation in the rat.

The males made enormous growth on rations I and J, some attaining a body weight of 375 gm in less than 5 months.

The role of lysine, valine and threonine in economy of food utilization (EFU)

This study was conducted on 24 weaned animals with initial weights of from 50 to 56 gm. They were divided into 4 groups of 6 animals each and the groups were given rations A, B, G and H, respectively. They were fed 7 gm daily each of the respective rations 6 times weekly and 14 gm on Saturdays. Each animal consumed the daily allotment of food completely without any waste. It will be noted from table 3 that on the same amount of food daily, 0.4% L-lysine in the ration produced 145% increased growth and 141% increase in economy of food utilization (EFU). There was no demonstrable effect of 0.7% DL-valine on EFU, but 0.5% DL-threonine in the presence of 0.4% L-lysine produced a *specific* effect of 208% increased growth and 206% increase in EFU. These same groups of animals were then fed ad libitum for the subsequent 6 weeks, with the following results: Lysine produced 460% increased growth and a 234% increase in EFU; valine + lysine produced 636% increased growth and a 320% increase in EFU; and threonine + lysine + valine produced 946% increased growth and a 431% increase in EFU.

DISCUSSION

The results of experiments of this type may have applications to peoples of low income levels who live largely on cereal grains as sources of calories and proteins. This includes a considerable majority of the people of the world, in all probability. The conversion of vegetable calories into animal calories has long been known to be inefficient. Only 15% of vegetable calories is recovered in producing milk,

7% in eggs, and 4% in beef (de Castro, '52). Hence plant proteins, consumed largely in cereal grains, cereal flours and beans, provide the cheapest sources of protein nitrogen for peoples with low income, notably in the Orient and in Latin America. If the cost of commercial production of amino acids can be reduced [4] and the resulting products can be made readily available, the application of the results of such studies as these could constitute a significant contribution toward improved nutrition for these poorly fed population groups throughout the world.

SUMMARY

A study was made, using the albino rat as the experimental animal, of the influence of additions of the amino acids, lysine, valine and threonine, and an extract from condensed fish solubles, on the efficiency of the proteins in vitamin-enriched milled wheat flour. It was found that the additions of such micronutrients changed the protein efficiency ratio of the proteins in white flour from 0.68 to 3.11. On the basis of similar nitrogen intake, the basal diet, containing proteins derived from milled wheat, supplemented with lysine, threonine, valine and an extract from condensed fish solubles, approximately equalled the biological value, from the standpoint of growth, of a ration containing proteins derived from dried non-fat milk solids.

Lysine and threonine were found to play a significant *specific* role in economy of food utilization, which was determined by controlled feeding experiments.

As little as 0.1 μg of vitamin B_{12} per animal per day, supplementing the ration containing lysine, was followed by appreciable increases of body weight in male rats, during a 12-week experimental period, but no noteworthy increases in growth were observed in the female rats on the vitamin B_{12}-supplemented ration. The increased growth in the male rats was due largely to greater food consumption.

[4] The cost of methionine production has already been reduced to the extent that it is being used economically in chicken feeds, and definite progress is being made in the economical commercial production of lysine.

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THE EFFECT OF ANTIBIOTICS ON THE METABOLISM OF CERTAIN B VITAMINS

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Recent reports (Linkswiler et al., '51; Lih and Baumann, '51; Sauberlich, '52) indicate that certain antibiotics stimulate the growth of rats receiving suboptimum amounts of thiamine, riboflavin, pantothenic acid or pyridoxine. It has been assumed that antibiotics, by suppressing intestinal organisms which deprive the host of limiting nutrients, enable more of the limiting vitamin to be absorbed by the host itself. Another explanation of the underlying mechanism could be the alteration of the intestinal flora by the antibiotic in such a way as to permit increased synthesis of the necessary vitamin. Through either mechanism antibiotics may exert a sparing action on certain B vitamins. If this view is correct, then rats receiving antibiotics would be expected to excrete larger amounts of these vitamins in their urine, and perhaps in their feces as well, and to accumulate larger amounts in their livers. To test this hypothesis the effect of antibiotics (aureomycin, terramycin, penicillin and streptomycin) on the metabolism of three vitamins of the B complex (thiamine, riboflavin and pantothenic acid) was studied.

MATERIALS AND METHODS

Male rats weighing 40 to 50 gm were placed on a basal diet of the following percentage composition: cornstarch 73,

vitamin-free casein 18, salts 4, and olive oil 5, plus the following vitamins, in milligrams per kilogram of ration: thiamine 2.0, riboflavin 3.0, pyridoxine 2.0, nicotinic acid 50, calcium pantothenate 20, choline chloride 1,000. Each rat received also 100 I.U. vitamin A and 4 I.U. vitamin D twice weekly. The levels of vitamins fed in the various limiting diets were as follows (milligrams per kilogram of ration): thiamine 1.0 and 0.25, riboflavin 1.0, and pantothenic acid 2.0. Food was offered ad libitum.

The antibiotics aureomycin, penicillin, terramycin and streptomycin were fed to different groups, being incorporated in the ration at a level of 50 mg per kilogram of diet.[1]

At the end of the third and of the 5th week of the experiment, urine and feces were collected from each rat during a three-day period. In the experiments on riboflavin and on pantothenic acid both feces and urine were examined; in the experiments on thiamine the excretion of this vitamin was determined in the feces only. In a second series of experiments the antibiotics were administered parenterally in amounts approximating the quantity eaten by the animals in the first series. Half a milliliter of an aqueous solution containing 0.5 mg of the antibiotic was injected subcutaneously daily. At the end of the 5th week all rats were killed and their livers analyzed for thiamine by the thiochrome method, or for riboflavin or pantothenic acid microbiologically (Association of Vitamin Chemists, '51).

RESULTS

The effect of orally administered antibiotics

Penicillin, aureomycin, terramycin and streptomycin were found to promote the growth of rats receiving suboptimum amounts of riboflavin or of pantothenic acid. In rats which

[1] The antibiotics used in this study were generously supplied by the Lederle Laboratories (aureomycin), Chas. Pfizer and Co. (terramycin), E. R. Squibb and Sons (streptomycin), and Teva, Middle East Chemical and Pharmaceutical Co., Jerusalem, Israel (penicillin).

were maintained on a diet limited in thiamine, only penicillin and terramycin stimulated growth, whereas aureomycin and streptomycin were without effect (table 1). However, when the full amounts of the vitamins were given, no effect of antibiotics on weight increase could be observed.

The excretion of the vitamins in urine and feces was also affected by the administration of antibiotics. Since similar values for urinary and fecal excretion of the vitamins were obtained during the two collection periods, the values are combined in table 1. The antibiotics increased the urinary excretion of riboflavin and pantothenic acid at all levels of vitamin intake, the only exception being the combination of penicillin and a high riboflavin intake. When aureomycin, streptomycin or terramycin was added to a diet low in pantothenic acid, and when penicillin was given with a diet low in riboflavin, there was a significant increase in the fecal excretion of these vitamins. Penicillin increased the level of the vitamins in the liver when added to diets poor in vitamins, but it was without effect when high levels of pantothenic acid or thiamine were fed. Aureomycin and terramycin likewise raised the vitamin concentration with diets low in thiamine, riboflavin or pantothenic acid, whereas streptomycin was effective only on the pantothenic acid and riboflavin concentrations in the liver.

It follows, therefore, that the antibiotics studied inhibit the suppression of growth of rats receiving limiting amounts of thiamine, riboflavin or pantothenic acid, except for aureomycin and streptomycin, which failed to stimulate the growth of rats kept on suboptimum amounts of thiamine. This growth-promoting effect is accompanied by an increase of both urinary excretion and concentration of the vitamins in the liver. Furthermore, even when growth is not affected by an insufficient supply of dietary vitamins, both aureomycin and penicillin increase either the urinary excretion or the liver level of riboflavin or pantothenic acid. None of the antibiotics, however, increased the amount of thiamine found in the liver.

TABLE 1

The effect of orally administered antibiotics on growth and on vitamin levels in feces, urine and liver
(Means and standard errors)

NO. OF RATS	VITAMINS	ANTIBIOTIC	WEIGHT INCREASE IN 5 WEEKS	VITAMIN IN URINE	VITAMINS IN FECES	VITAMIN IN LIVER, μG	
						Per gram	Total
	mg/kg diet		*gm*	*μg/day*	*μg/day*		
				Thiamine			
12	2	...	91 ± 2.5		0.88 ± 0.05	1.45 ± 0.13	8.64 ± 0.68
9	2	Penicillin	89 ± 3.8		0.88 ± 0.07	1.45 ± 0.15	9.33 ± 0.62
13	2	Aureomycin	83 ± 5.1		0.73 ± 0.08	1.23 ± 0.11	6.95 ± 0.60
12	1	...	37 ± 7.0		0.91 ± 0.11	0.26 ± 0.05	0.85 ± 0.12
12	1	Penicillin	84[1] ± 2.9		0.65 ± 0.08	0.46[1] ± 0.03	2.88[1] ± 0.23
10	1	Aureomycin	38 ± 3.3		0.71 ± 0.08	0.26 ± 0.03	1.04 ± 0.13
12	1	Streptomycin	39 ± 2.4		0.66 ± 0.09	0.31 ± 0.06	1.03 ± 0.09
12	1	Terramycin	69[1] ± 3.9		0.85 ± 0.14	0.30 ± 0.04	1.49 ± 0.22
11	0.25	...	5 ± 2.2		0.27 ± 0.09	0.11 ± 0.02	0.28 ± 0.06
6	0.25	Penicillin	15[3] ± 3.6		0.33 ± 0.05	0.56[4] ± 0.15	1.64[4] ± 0.48
6	0.25	Aureomycin	7 ± 3.5		0.41 ± 0.05	0.27[2] ± 0.06	0.57[4] ± 0.05
10	0.25	Streptomycin	4 ± 2.6		0.25 ± 0.02	0.14 ± 0.03	0.34 ± 0.07
6	0.25	Terramycin	12[2] ± 2.7		0.32 ± 0.04	0.33[1] ± 0.03	0.89[1] ± 0.07
				Riboflavin			
12	3	...	91 ± 2.5	7.6 ± 0.53	11.2 ± 0.69	30.8 ± 1.17	187 ± 10.9
9	3	Penicillin	89 ± 3.8	7.8 ± 0.69	9.9 ± 0.76	35.0[3] ± 1.77	229[2] ± 13.3
13	3	Aureomycin	83 ± 5.1	10.5 ± 0.90	10.6 ± 0.64	33.8 ± 4.27	189 ± 25.4
12	2	...	33 ± 2.9	1.7 ± 0.12	5.1 ± 0.57	16.5 ± 1.35	72 ± 6.4
12	2	Penicillin	56[1] ± 2.9	2.1[3] ± 0.14	7.0[3] ± 0.71	24.4[1] ± 1.19	103[1] ± 4.2
12	2	Aureomycin	59[1] ± 5.0	2.2[3] ± 0.18	6.1 ± 0.36	24.8[4] ± 2.34	103[4] ± 8.7
11	2	Streptomycin	50[1] ± 4.1	2.3[3] ± 0.23	5.6 ± 0.65	23.4[1] ± 1.43	96[4] ± 5.0
12	2	Terramycin	53[1] ± 3.7	2.1[3] ± 0.18	5.7 ± 0.57	24.1[1] ± 1.51	103[1] ± 4.6
				Pantothenic acid			
12	20	...	91 ± 2.5	59.9 ± 5.0	17.5 ± 1.8	58.3 ± 5.4	358 ± 45
9	20	Penicillin	89 ± 3.8	100.5[3] ± 15.1	20.4 ± 2.3	54.5 ± 4.5	360 ± 38
12	20	Aureomycin	83 ± 5.1	92.3[3] ± 13.7	21.3 ± 2.0	50.4 ± 6.2	328 ± 36
8	2	...	35 ± 1.6	2.3 ± 0.24	11.2 ± 1.4	50.8 ± 1.5	197 ± 10
6	2	Penicillin	64[1] ± 3.5	3.4[4] ± 0.28	12.7 ± 0.7	62.7[1] ± 2.7	297[1] ± 11
6	2	Aureomycin	59[1] ± 2.3	3.9[4] ± 0.44	15.5[4] ± 1.5	56.6[2] ± 2.2	271[4] ± 20
6	2	Streptomycin	67[1] ± 4.1	3.6[4] ± 0.40	16.1[2] ± 1.5	58.6[2] ± 2.9	307[1] ± 28
6	2	Terramycin	72[1] ± 5.1	7.0[1] ± 0.82	16.8[4] ± 1.2	50.8 ± 2.7	305[1] ± 27

[1] Significant at 0.1% level.
[2] Significant at 2% level.
[3] Significant at 5% level.
[4] Significant at 1% level.

The effect of parenterally-administered antibiotics

The vitamin-sparing effect of antibiotics may either be brought about by their effect on the intestinal flora or it may be due to a direct action on tissue metabolism. In order to study this question, a second series of experiments was conducted in which the animals received the antibiotics by daily subcutaneous injections. In the experiments with

TABLE 2

The effect of subcutaneously administered antibiotics on growth and vitamin content of liver

(Means and standard errors)

NO. OF RATS	VITAMIN STUDIED	VITAMIN	ANTIBIOTIC	WEIGHT INCREASE IN 5 WEEKS	VITAMIN IN LIVER, μG	
					Per gram	Total
		mg/kg diet		*gm*		
5	Thiamine	1	.....	44 ± 4.6	0.26 ± 0.06	1.07 ± 0.25
10	Thiamine	1	Terramycin	53 ± 4.3	0.28 ± 0.02	0.99 ± 0.10
10	Thiamine	1	Penicillin	54 ± 5.0	0.33 ± 0.03	1.43 ± 0.16
5	Riboflavin	2	.....	48 ± 6.9	23.4 ± 2.21	91 ± 1.6
10	Riboflavin	2	Aureomycin	53 ± 6.7	25.9 ± 1.16	100 ± 7.6
10	Riboflavin	2	Penicillin	47 ± 6.1	28.3 ± 2.09	106 ± 9.1
5	Pantothenic acid	2	.....	44 ± 6.8	37.5 ± 1.59	173 ± 7.0
10	Pantothenic acid	2	Aureomycin	53 ± 3.9	36.8 ± 2.53	186 ± 11.2
10	Pantothenic acid	2	Penicillin	55 ± 5.4	33.3 ± 2.17	170 ± 13.3

riboflavin and pantothenic acid, aureomycin and penicillin were given, and in the experiments with thiamine, terramycin and penicillin. The results obtained are shown in table 2.

It follows from the results shown in table 2 that none of the antibiotics when administered subcutaneously exhibited a significant growth-promoting effect on rats maintained on suboptimum amounts of thiamine, riboflavin or pantothenic acid. Nor did they significantly raise the level of these vitamins in the liver. These results do not support the assumption

of a direct action of the antibiotics on the vitamin economy of the body. The antibiotics seem, therefore, to exert their influence mainly on the intestinal bacteria.

DISCUSSION

The present study shows that some antibiotics when added to the diet appear to have an effect upon the requirement of young rats for certain B vitamins. All the studied antibiotics appeared to be effective in riboflavin and pantothenic acid metabolism, but their effect in thiamine metabolism was less distinct and streptomycin was without any effect on this vitamin. Whereas no growth-stimulating effect of antibiotics was observed in rats receiving a completely vitamin-supplemented diet, penicillin increased the liver concentration of riboflavin, aureomycin the urinary excretion of riboflavin, and both penicillin and aureomycin the urinary excretion of pantothenic acid. Our experiments in which antibiotics were administered parenterally demonstrate that this effect is related to alteration of the intestinal flora. These findings are in agreement with our observation that feeding aureomycin, streptomycin and terramycin in a diet low in pantothenic acid, and penicillin in a diet low in riboflavin, significantly raised the fecal output of these vitamins. Better intestinal absorption may explain the cases where, despite increases in liver concentration of vitamins or urinary excretion, there was no increase in the fecal excretion of the vitamins. Emerson and Obermeyer ('45) and Obermeyer et al. ('45) found that the output of thiamine and of riboflavin in the feces of rats depends rather on the amount of food consumed and the quantity of feces excreted than on the intake of these vitamins.

The present results with antibiotics are in marked contrast to previous results with sulfonamides, where requirements for certain vitamins were increased by their presence in the diet (Najjar and Barrett, '45; Elvehjem, '48). They are, however, in general agreement with the findings reported by Linkswiler et al. ('51), Daft and Schwarz ('52), Lih and Baumann ('51) and Sauberlich ('52). The latter authors, studying the

effect of antibiotics on the growth of rats fed diets deficient in certain B vitamins, found a marked stimulation by penicillin in thiamine deficiency, by aureomycin in riboflavin and pantothenic acid deficiencies, and by streptomycin in pantothenic acid deficiency. Smaller weight gains were induced by streptomycin in thiamine and riboflavin deficiencies, and by terramycin in riboflavin deficiency. The effect of penicillin in riboflavin deficiency was more marked in the study of Lih and Baumann than in that of Sauberlich, whereas the latter noted a more distinct influence of aureomycin in thiamine deficiency than Lih and Baumann, who found that terramycin was without effect in thiamine deficiency. In our experiments, terramycin was quite active, whereas streptomycin was inactive in thiamine subdeficiency. The 4 antibiotics studied had more or less the same effect in pantothenic acid and in riboflavin subdeficiencies. This difference may be due to different sources of carbohydrates in the diet. Both Sauberlich and Lih and Baumann used sucrose and dextrin in their diets, whereas we used starch.

The effect of oral administration of streptomycin on the urinary excretion of B vitamins in man has been studied by Sarett ('52). Inconstant changes were observed for folic acid, thiamine, riboflavin and vitamin B_6. However, biotin excretion was markedly reduced and excretion of methylnicotinamide and of the 6-pyridone of methylnicotinamide appeared to be slightly reduced.

Our findings that parenterally administered antibiotics exert no effect on the vitamin content of the liver agree well with the results of Whitehall et al. ('50), McGinnis et al. ('50) and Briggs ('50).

While our results appear to demonstrate that the vitamin-sparing effect of antibiotics is caused by their influence on the intestinal flora, they do not provide an explanation of the mechanism by which this effect is brought about. This may be either through decreasing the number of bacteria which absorb vitamins or through alteration of the flora so as to

increase the synthesis of vitamins. This problem is now under study.

SUMMARY

1. The effect of penicillin, aureomycin, streptomycin and terramycin upon the metabolism of thiamine, riboflavin and pantothenic acid was studied in rats.

2. The addition of these antibiotics to the diet (50 mg/kg) caused a marked stimulation of the growth of rats fed diets low in riboflavin and pantothenic acid. A similar effect was observed with penicillin and terramycin added to a low-thiamine diet, but not with aureomycin and streptomycin. The inclusion of the antibiotics in a completely vitamin-supplemented diet had no growth-promoting effect.

3. Growth stimulation was generally associated with increased urinary excretion and a greater liver level of vitamins. Penicillin increased the riboflavin content of the liver, aureomycin the riboflavin excretion in urine, and both aureomycin and penicillin increased the urinary excretion of pantothenic acid when given with non-limiting amounts of these vitamins. Aureomycin, streptomycin and terramycin in a diet low in pantothenic acid, and penicillin added to a diet low in riboflavin, raised the fecal output of these vitamins.

4. Subcutaneous injections of aureomycin and penicillin in rats fed diets low in riboflavin or pantothenic acid, and of penicillin and terramycin in rats on a low-thiamine diet, had no effect on growth or on the accumulation of these vitamins in the liver.

5. It is concluded that the observed sparing effect of antibiotics on the requirement of young rats for certain B vitamins is mainly due to an alteration of the intestinal flora.

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THE DESTRUCTION OF B VITAMINS BY CERTAIN SALT MIXTURES IN PURIFIED RATIONS

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THREE FIGURES

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Lyman and Elvehjem ('51) have recently reported the fact that vitamin B_1 deficiency can be produced in rats by feeding them a synthetic ration, even when the ration contains as much as 0.2 mg % of this vitamin. Chemical analyses disclosed that up to 80% of the thiamine added to such rations as part of a stable vitamin mix could be destroyed within two weeks. It was suggested that the decrease in active thiamine might have been brought about by components of the salt mixture used. The following observations, made some years ago, may be of help in elucidating the exact mechanism of this remarkable destruction, which deserves special attention wherever rats are used for biological vitamin assays.

In the laboratory at Delft a purified ration B (Rombouts and Querido, '46), to which a salt mixture, prepared as described in the U. S. Pharmacopoeia ('36), was added, had been successfully used during several years for rearing albino rats for vitamin assay purposes. When the attempt was made to replace this salt mixture with the one described by Phillips and Hart ('35), nearly all rats died a premature death, with symptoms which were partly unspecific, but partly, however,

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resembled those of a thiamine deficiency combined with a vitamin B_6 deficiency.

Rats which were fed the ration prepared some weeks previously with the salt mixture of Phillips and Hart grew slowly and their total gain in weight usually did not surpass 30 gm. After about three weeks the body weight decreased until death followed in about 6 weeks. Most animals produced black and unguinous excrements in the second week of the experiment, but in most cases the feces turned normal again in the course of another fortnight. Many animals showed a distinct uptilted nose. The fur became shaggy. Later the majority of the animals developed a mild wet dermatitis and excreted porphyrin, apparently from the lachrymal glands and the mucous membranes of the nose, resulting in a reddish brown fouling of the snout and whiskers. "Spectacled eyes," caused by falling hairs around the eyelids, was frequently observed. During the last days of their life the animals avoided moving; they sat with arched backs in a corner of the cage. The edematous soles of the feet were apparently very painful. When such rats were lifted by the tail they often developed convulsions, resulting in fast twisting motions. In most cases a distinct leukopenia was observed. Hemoglobin and erythrocyte numbers remained normal.

Both salt mixtures were apparently adequate sources of the essential minerals, since rats developed equally well when either one was added to the purified ration shortly before feeding the animals. However, when the ration supplemented with the salt mixture of Phillips and Hart was stored for two weeks or more, some factor, or factors, essential for rat growth was destroyed during that period. The same purified ration, when supplemented with the U.S.P. salt mixture instead, could be stored at room temperature during at least 6 weeks without losing its nutritive adequacy.

The salt mixtures to be discussed are as follows:

1. The U.S.P. ('36) salt mixture. This is prepared as follows: 111.1 gm of citric acid are dissolved in a sufficient quantity of hot distilled water and the solution added to a

mixture of 134.8 gm calcium carbonate, 28.9 gm magnesium carbonate (40% MgO), 34.2 gm anhydrous sodium carbonate, and 141.3 gm potassium carbonate (dried at 180°). Subsequently 20 mg potassium iodide, 117 mg $MnSO_4 \cdot 4\ H_2O$, 62 mg sodium fluoride, and 44 mg potassium alum, all of which have been previously dissolved in distilled water, are added. Then 7.44 gm ferric citrate (17.5% Fe) dissolved in 148.3 gm hydrochloric acid (36%) are added. Nine and six-tenths grams sulfuric acid (96%) are hereupon diluted with distilled water, 119.3 gm phosphoric acid (86.5%) added, and this whole acid mixture added to the mixture previously prepared and the whole stirred until effervescence ceases. The final mixture is evaporated to dryness in a current of air at 90° to 100°, and the resulting product reduced to a fine powder.

2. The salt mixture proposed by Phillips and Hart ('35). This is made up by mixing 600 gm $CaCO_3$, 845 gm $K_2HPO_4 \cdot 3\ H_2O$, 190 gm $CaHPO_4 \cdot 2\ H_2O$, 204 gm $MgSO_4 \cdot 7\ H_2O$, 335 gm NaCl, 55 gm $Fe(C_6H_5O_7)_2 \cdot 6\ H_2O$, 1.6 gm KJ, 0.7 gm $MnSO_4 \cdot 4\ H_2O$, 0.5 gm $ZnCl_2$, and 0.6 gm $CuSO_4 \cdot 5\ H_2O$.

3. The salt mixture of Hegsted et al. ('41) used as salt mixture IV by Lyman and Elvehjem ('51). It is composed of 600 gm $CaCO_3$, 645 gm K_2HPO_4, 150 gm $CaHPO_4 \cdot 2\ H_2O$, 204 gm $MgSO_4 \cdot 7\ H_2O$, 335 gm NaCl, 55 gm $Fe(C_6H_5O_7)_2 \cdot 6\ H_2O$, 1.6 gm KJ, 1.0 gm $MnSO_4 \cdot 4\ H_2O$, 0.5 gm $ZnCl_2$, and 0.6 gm $CuSO_4 \cdot 5\ H_2O$.

EXPERIMENTAL

A batch of 6 kg of the purified ration B (Rombouts and Querido, '46), from which, however, the salts were omitted, was divided into three equal portions which thereupon were completed with: (a) 40 gm of the U.S.P. salt mixture; (b) 20 gm of the U.S.P. salt mixture and 20 gm of the Phillips and Hart salt mixture; and (c) 40 gm of the Phillips and Hart salt mixture. Three groups of 12 young, weanling rats with a body weight of 34 gm were kept on the above diets and their average growth was recorded (fig. 1).

Of food (a) the total amount made was consumed in 16 days. At that time the experiment was continued with a freshly prepared portion of food of the same composition. There is a slight indication that this had a favorable influence on the growth rate, but the difference in the slopes of the growth curves is not significant.

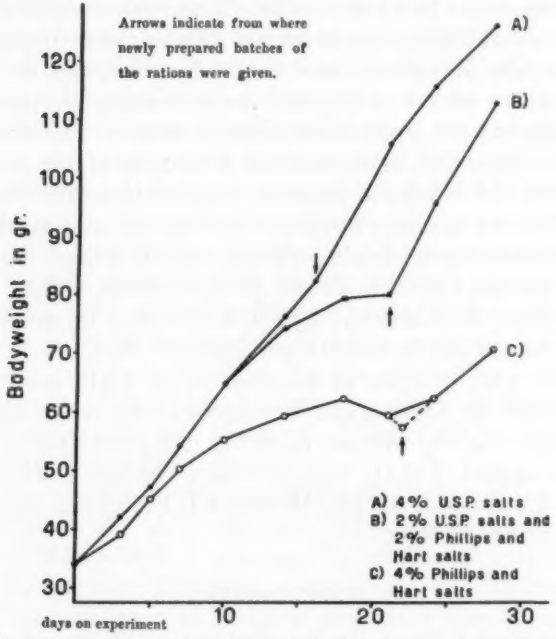

Fig. 1 Comparative growth of rats fed ad libitum rations containing different salts. Arrows indicate when newly prepared batches of the rations were given.

The 12 animals kept on food (b) consumed somewhat less than those of the previous group and they finished the original batch of food in 20 days. At that time growth had almost come to a complete standstill, but feeding a freshly made portion of the same diet restored normal growth at once.

The rats kept on food (c) began to lose weight after 18 days from the onset of the experiment. Although the originally prepared amount of food was not yet completely finished even at the 22nd day, a freshly prepared portion was given from that day onward. Growth was restored immediately, the daily gain in weight remaining, however, suboptimum.

A striking difference between the U.S.P. salt mixture and that of Phillips and Hart is that the latter contains zinc chloride and copper sulfate. When equivalent amounts of $ZnCl_2$ and $CuSO_4$ were added to the U.S.P. salts, and zinc and copper were omitted from the Phillips and Hart mixture, this modified U.S.P. mixture still gave optimum growth when used in the purified diet, whereas the use of the modified Phillips and Hart salts still resulted in the early death of most animals with the symptoms described above.

The possibility of a thiamine deficiency, judged by the observed symptoms, was early taken into account. Since it is common knowledge that stability of thiamine is greatest with low pH values, it seemed worthwhile to check as to whether the different influences of the salt mixtures on the adequacy of the same basic diet could be explained by a difference in acidity. A 2% suspension of the U.S.P. salt mixture in water had a pH of 5.4, whereas a 2% suspension of the Phillips and Hart mixture in water had a pH of 6.3. Although from these pH values it was hardly possible to expect the explanation of the inactivation of the thiamine, a watery suspension of the Phillips and Hart salt mixture was acidified by means of phosphoric acid until, after redrying, a 2% suspension in water had a pH of 5.4. By this addition of phosphoric acid the Ca/P ratio, which is 19:10 in the original formula, was altered to 10:7. In a similar way the U.S.P. salt mixture, which has a Ca/P ratio of 10:6, was made more alkaline by means of a sodium hydroxide solution until, after redrying, a 2% suspension in water had a pH of 6.5.

Two groups of young, male rats were kept on the purified ration completed with one of the two salt mixtures of which

the acidity had been altered as described above. Growth on the diet with the U.S.P. salts of pH 6.5 was still optimum, the average daily gain in body weight being 3.7 gm. When the acidified Phillips and Hart salt mixture was used instead, growth was much improved when compared with the growth on the original formula of the same salt mixture. The animals reached a body weight of 120 gm, with an average daily gain in weight of 2.6 gm, which is still well below the optimum.

In the next experiment three groups of 8 young male rats each were kept on the purified diet with a 4% addition of the Phillips and Hart salt mixture of the original formula. Group I had cod liver oil and adequate amounts of thiamine, riboflavin, pyridoxine, niacin, Ca-pantothenate and choline mixed with the food. For group II only the B vitamins were mixed with the food, whereas the cod liver oil was given orally twice a week. For group III the cod liver oil was mixed with the food, and a mixture of a solution of the B vitamins was given orally twice a week. There was no difference in the development of the rats between groups I and II. The maximum body weight reached in these groups was not more than 66 gm and most animals died within 5 weeks. In group III, however, growth was optimum. The average daily gain in weight was 3.5 gm until, after one month, a body weight of nearly 140 gm was reached. Obviously the Phillips and Hart salt mixture destroyed only one or more of the 6 B vitamins with which it was mixed in the ration and had no detrimental effect upon vitamins A and D.

In order to elucidate which B vitamins were affected, the following experiments were carried out. Seven groups of 12 young rats each were fed the purified ration containing the usual mixture of the 6 B vitamins and the Phillips and Hart salts. The whole batch of food was prepared some weeks before the onset of the experiment in order to enable the minerals to react with the vitamins. Apart from this ration fed ad libitum the animals of the different groups were given orally, with the aid of a stomach tube, one B vitamin extra, as listed below:

Group 1: three times a week 50 µg thiamine HCl.
Group 2: three times a week 60 µg riboflavin.
Group 3: three times a week 60 µg pyridoxine HCl.
Group 4: three times a week 6 mg niacinamide.
Group 5: three times a week 0.4 mg Ca-pantothenate.

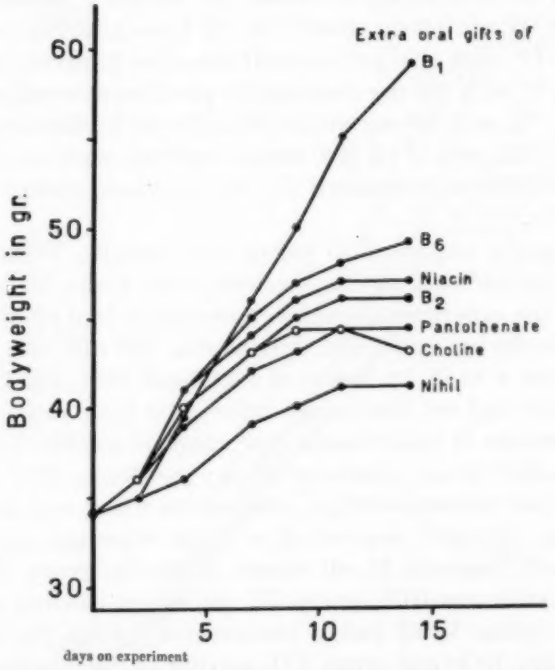

Fig. 2 Comparative growth of rats fed ad libitum rations containing 6 B vitamins mixed with the food. Moreover, one of these B vitamins was given extra by mouth, as indicated.

Group 6: three times a week 6 mg choline chloride.
Group 7: no extra (control).

The amount of food prepared for this experiment was large enough to feed all 84 animals during a fortnight but did not last for a longer period. The growth rates of the different groups are presented in figure 2.

In another experiment a batch of the purified ration was mixed with the Phillips and Hart salts, whereas the 6 B vitamins were omitted. The batch was thereupon divided into 7 equal groups which were subsequently enriched as follows:

Group I, with 2 mg thiamine HCl per kilogram ration.
Group II, with 2.5 mg riboflavin per kilogram ration.
Group III, with 3 mg pyridoxine HCl per kilogram ration.
Group IV, with 10 mg Ca-pantothenate per kilogram ration.
Group V, with 300 mg niacinamide per kilogram ration.
Group VI, with 300 mg choline chloride per kilogram ration.
Group VII, with the 6 B vitamins together, each one in the concentration mentioned for the previous groups separately.

Each group contained 10 young rats (weight, 38 gm) and the amount of food, again prepared some weeks before the onset of the experiment, was large enough to feed all animals ad libitum during a period of three weeks. The rats were given three times a week, by means of a stomach tube, the B vitamins which had not been mixed before into their ration. The extra amounts of each vitamin thus supplied were as follows: thiamine-HCl 50 μg; riboflavin 60 μg; pyridoxine-HCl 60 μg; calcium pantothenate 0.4 mg; niacinamide 6 mg; and choline-HCl 6 mg. Group I received all of these vitamins except the thiamine-HCl; group II, all except riboflavin; group III, all except pyridoxine-HCl; group IV, all except calcium pantothenate; group V, all except niacinamide; group VI, all except choline-HCl; and group VII, serving as the negative control, received none of these vitamins.

The growth rates of the different groups are presented in figure 3.

DISCUSSION

From the results of the experiments it must be concluded that the salt mixture of Phillips and Hart has a destructive effect on the adequacy of synthetic B vitamins added to purified rations. It is not possible to explain the facts observed

by a specific action on one single vitamin, though thiamine is obviously most affected. Extra oral supplements of thiamine give better results than a separate supply of any other of the 6 B vitamins used in this study. However, extra B_1

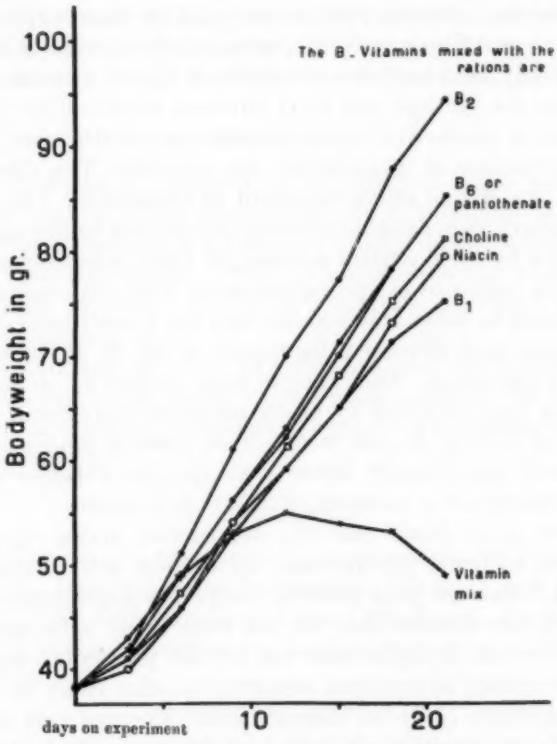

Fig. 3 Comparative growth of rats fed ad libitum rations containing only one B vitamin mixed with the food, as indicated. The other 5 B vitamins were given separately by mouth.

does not restore growth to the same rate as does a mixture of all the 6 B vitamins. Moreover, several of the other B vitamins may, to some extent, also bring about a retardation in the development of deficiency symptoms when administered

separately (fig. 2). Riboflavin activity is apparently hardly affected by the mineral constituents of the salt mixture (fig. 3). It is not unlikely that complicated interrelationships exist between the members of the vitamin B complex and that reciprocal effects of sparing or protecting action are responsible for the intricate picture revealed by these experiments.

Lyman and Elvehjem ('51), using a salt mixture of Hegsted et al. ('41), of which the composition shows a striking similarity to the Phillips and Hart mixture, disclosed by chemical analyses a protective action of succinylsulfathiazole against the destruction of thiamine by the minerals. The mechanism of that stabilizing effect could not be elucidated. The American authors were capable of restoring growth to the same rate as shown by their control animals by daily injections of 20 μg B_1. This seems to be in disagreement with our observations, but it must be taken into account that the growth rate obtained by Lyman and Elvehjem by means of the B_1 injections was about 2 gm a day. This gain in body weight is virtually the same as that obtained by us by means of extra oral supplements of 150 μg B_1 per week. This growth rate was, however, still significantly lower than the one reached with an extra supply of a mixture of the 6 B vitamins.

There is no doubt that the destructive action upon thiamine is, at least, precipitated by certain salt mixtures, although it has not been possible, as yet, to explain the mechanism of the destruction. In the case dealt with here it is obviously not an effect exerted by the particular nature of certain metals, as has been observed in other cases by De Ritter and Rubin ('47) for reduced iron. The two salt mixtures used in our experiments both contain iron only in the form of ferric salts. They differ essentially in their copper and zinc content, but an exchange of these two elements does not alter the salts' influence on the vitamin B complex. The concentration of hydrogen ions, with which the salt mixtures are in equilibrium when suspended in water, shows a certain correlation with the stability of the B vitamins as measured by the growth of rats. This may be of importance if one keeps

in mind that the rations used in these experiments contained an appreciable amount of moisture; the B vitamins were always mixed with the other food components in the form of a solution of the vitamins in some water in order to ensure an even distribution. Moreover, the rations were rather hygroscopic by reason of their high sugar content. A possibility, as yet unexplored experimentally, is that this destruction of vitamins results from the creation of unfavorable Redox potentials.

SUMMARY

The salt mixture proposed by Phillips and Hart may exert a detrimental effect upon synthetic B vitamins in purified rations. Thiamine is apparently most, riboflavin least, affected. This destructive effect was not observed when the rations were prepared with a salt mixture described in the U.S. Pharmacopoeia.

The destructive action of the Phillips and Hart salt mixture is not due to its $ZnCl_2$ or its $CuSO_4$ content. Its higher pH, when in a watery suspension, seems to be partly responsible for the effect.

ACKNOWLEDGMENT

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DIFFERENCE IN THE CARIES-PRODUCING EFFECTS OF TWO PURIFIED DIETS CONTAINING THE SAME AMOUNT OF SUGAR

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TWO FIGURES

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In a previous study (Haldi and Wynn, '52), the incidence and extent of dental caries in Wistar strain albino rats fed a purified high sucrose diet were found to be considerably less than had been reported by Sognnaes ('48) for rats of the Long Evans strain fed a similar purified diet. Both diets, which were adequate for growth and for the maintenance of normal health, contained the same amount of sucrose, but they differed quantitatively in some details with respect to their fat, protein, mineral and vitamin contents.

Without further investigation it is impossible to state conclusively whether the different results obtained in the two laboratories were due to differences in the composition of the Harvard and Emory diets or to other experimental conditions. Genetic factors may have influenced the experimental findings, since the rats in the two experiments were of different strains. Recent studies (Schweigert, Shaw, Elvehjem and Phillips, '45; McClure, '51; Holmes, '52) provide ample evidence of a strain difference in susceptibility to dental caries. Dissimiliar conditions in the two laboratories, such as

caging, temperature, and humidity control, and also a difference in the drinking water of the two localities could likewise conceivably have contributed to the outcome of the experiments. In view of these considerations, it was deemed advisable to conduct further experiments, maintaining all the variables constant except the composition of the two diets fed the test and control animals.

EXPERIMENTAL

In order to obtain comparative observations on two distinct species, two experiments were conducted: one on albino rats of the Wistar strain, the other on cotton rats. All the animals in both experiments were raised in the colonies maintained in the Emory laboratories and were housed therein throughout the experimental periods. The environmental conditions were therefore the same for all the animals. Feeding was permitted ad libitum.

Twenty-four pairs of albino rats were selected at weaning and were placed immediately on the Emory and Harvard diets, of exactly the same composition as those employed in the previous experiments (Haldi and Wynn, '52; Sognnaes, '48). The composition of these diets is given in table 1. These rats had been found to have a fairly high resistance to caries when fed a generally adequate, purified diet from weaning time, even if the sugar content of the diet was as high as 64%. In order to accelerate the carious process, they were therefore surgically desalivated by a block dissection method described elsewhere (Haldi, Wynn, Shaw and Sognnaes, '53).

When the animals had been fed the diets for 80 days, they were sacrificed and the heads boiled in water to loosen adherent tissue. The molar teeth were thoroughly cleaned and then examined under a dissecting microscope for carious lesions. Examination and scoring were done in both the Emory and Harvard laboratories. A comparison of the scores made by the different observers showed the same relative differences on the two diets, although there was some divergence in the absolute scores because of the different scoring meth-

TABLE 1

Composition of the Emory and Harvard high sucrose diets

CONSTITUENTS	EMORY DIET	HARVARD DIET
	%	%
Sucrose	64	64
Casein	20	23
Hydrogenated vegetable oil	8	..
Corn oil	.	5
Yeast and liver extract	4	..
Liver concentrate	.	4
Salt mixture	4	4

CONTRIBUTED BY SALT MIXTURE

	Emory	Harvard
Potassium (%)	0.72	0.45
Calcium (%)	0.60	0.53
Sodium (%)	0.40	0.44
Phosphorus (%)	0.38	0.55
Magnesium (%)	0.053	0.033
Chloride (%)	0.18	0.76
Iron (p.p.m.)	480	150
Sulfur (p.p.m.)	310	480
Manganese (p.p.m.)	78	50
Copper (p.p.m.)	10	15
Zinc (p.p.m.)	4	30
Iodide (p.p.m.)	1.5	20
Aluminum (p.p.m.)	0.16	..
Fluoride (p.p.m.)	0.1	0
Cobalt (p.p.m.)	..	0.65

VITAMINS

	Emory [1]	Harvard [2]
Choline chloride (p.p.m.)	3,000	1,000
α-Tocopherol (p.p.m.)	300	..
Inositol (p.p.m.)	130	1,000
Niacin (p.p.m.)	32	25
Calcium pantothenate (p.p.m.)	25	20
Thiamine·HCl (p.p.m.)	24	3.5
Riboflavin (p.p.m.)	24	3.5
Pyridoxine·HCl (p.p.m.)	10	3.5
β-Carotene (p.p.m.)	3	30 (U.S.P. U./gm)
Folic acid (p.p.m.)	1	..
Biotin (p.p.m.)	012	..
p-Aminobenzoic acid (p.p.m.)	..	300
A concentrate (I.U./gm)	10	..
Viosterol (U.S.P. units/gm)	3	1.5
Linoleic acid (p.p.m.)	500	..

[1] These values include the vitamins contained in the yeast and liver extract as supplied by the manufacturer.

[2] These values do not include the vitamins contained in the liver concentrate, as these values were not available.

ods that were followed. The scores presented in table 2 were those recorded in the Harvard laboratories. The averages were drawn from the combined data on the teeth of the males and females, as no sex difference in the albino rat has been observed in respect to susceptibility to dental caries (Haldi, Wynn, Shaw and Sognnaes, '53).

Litter-mate cotton rats in groups of three were selected at weaning whenever possible. In some instances only one or two animals could be obtained from a litter. When there were three in a litter, one was placed on the Emory, one on the Harvard high sucrose diet, and another on a stock diet which consisted solely of a commercial animal food.[1] When only two animals were available, they were assigned to the different

TABLE 2

Dental caries in albino rats fed the Emory and Harvard diets[1]

DIET	NUMBER OF ANIMALS	AVERAGE NUMBER OF CARIOUS MOLARS	AVERAGE NUMBER OF CARIOUS LESIONS	AVERAGE CARIES SCORE
Emory	24	5.1 (± 0.6)	7.3 (± 1.0)	24.9 + (± 3.9)
Harvard	24	10.2 (± 1.4)	21.8 (± 1.1)	83.8 + (± 5.4)

[1] The figures in brackets are the standard errors of the means (S.E.M.)

diets at the discretion of the experimenter. The cotton rats were not desalivated, as their caries susceptibility was high enough to produce an appreciable amount of caries on a high sucrose diet in a relatively short period of time without this procedure. After 100 days they were sacrificed and the jaws removed and prepared for examination. The system employed in the evaluation of the number and extent of the carious lesions in cotton rats has been described elsewhere (Shaw, Schweigert, McIntire, Elvehjem and Phillips, '44). The data for the examinations which were made by one of us (J. H. S.) are presented in table 3. Since there is no sex difference in susceptibility to dental caries in the cotton rat (Shaw, unpublished data), the averages were obtained from the combined data on males and females.

[1] Purina laboratory chow.

RESULTS

Tables 2 and 3 show that the two synthetic diets employed in these experiments, both of which contained the same amount of sugar, were markedly different in their cariogenic properties. In the albino rats the Harvard diet produced three times as many carious lesions and a caries score over three times as great as that which was caused by the Emory diet. Similarly, in the cotton rats both the number of carious lesions and the caries score were approximately two to three times higher on the Harvard diet. These differences in the caries score on the two diets were statistically highly significant. The incidence of caries in the cotton rats on the stock diet was much

TABLE 3

Dental caries in cotton rats fed the Emory and Harvard diets[1]

DIET	NUMBER OF ANIMALS	AVERAGE NUMBER OF CARIOUS MOLARS	AVERAGE NUMBER OF CARIOUS LESIONS	AVERAGE CARIES SCORE
Emory	17	3.6 (± 0.4)	7.0 (± 0.8)	15.4 + (± 2.1)
Harvard	20	6.0 (± 0.7)	13.4 (± 1.7)	41.9 + (± 7.6)
Stock diet[2]	17	1.0 (± 0.6)	2.0 (± 0.1)	3.8 + (± 2.5)

[1] The figures in brackets are the standard errors of the means (S.E.M.).
[2] Purina Laboratory Chow.

lower than on either purified diet, with carious lesions in only 5 of 17 of these animals.

In addition, detailed comparison of the individual data revealed that each of the 24 albino rats fed the Harvard diet had a higher score than its litter mate on the Emory diet. When the lesions were graded from one to 4 according to their severity (Haldi and Wynn, '52), there were 154 lesions with a score of 4 in the albino rats fed the Harvard diet, whereas there were no lesions with a score greater than two in the teeth of the rats on the Emory diet. Photographs in figures 1 and 2 present pictorial evidence of the difference in the severity of caries on the two rations. Illustration of the most severe lesions obtained on the two diets is presented in fig-

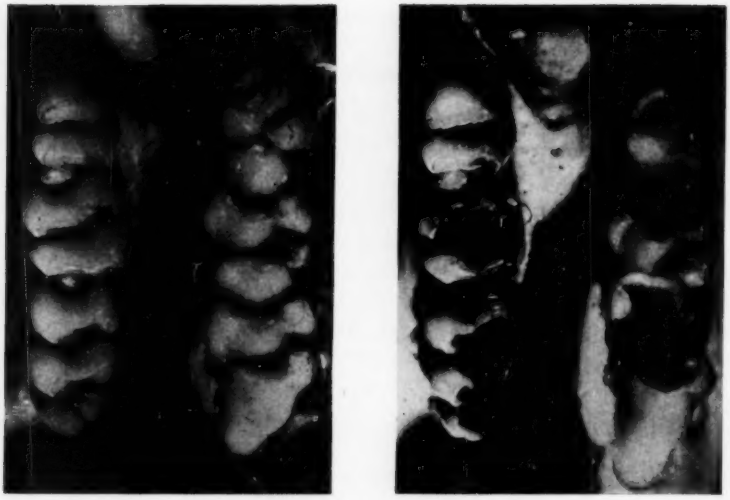

Fig. 1 Examples of most severe caries.
Left — Lower and upper left quadrants of animal on the Emory diet.
Right — Lower and upper left quadrants of animal on the Harvard diet.

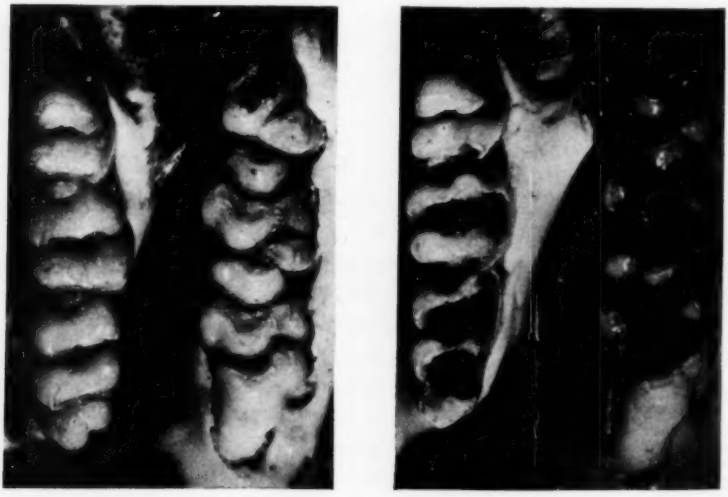

Fig. 2 Examples of caries in litter mates.
Left — Lower and upper left quadrants of animal on Emory diet.
Right — Lower and upper left quadrants of animal on Harvard diet.

ure 1, while a typical contrast of the teeth of litter mates is shown in figure 2.

DISCUSSION

From these experiments it can be concluded that dietary factors other than sugar account at least in part for the previously noted difference in cariogenicity between the Emory and Harvard diets (Haldi and Wynn, '52).

Our findings are in agreement with those of Constant, Phillips and Elvehjem ('52), who also showed that the cariogenicity of diets is not entirely predictable on the basis of their sugar content. In their experiments a synthetic diet containing 67% sucrose was less cariogenic than a diet consisting of 50% oatmeal, 32% dried whole milk and 18% sucrose. On the high sugar diet there were 38% fewer carious lesions and a 50% reduction in the severity of the lesions. Similar results had previously been obtained in the same laboratory (Zepplin, Smith, Parsons, Phillips and Elvehjem, '50). Natural diets containing 17% sucrose and resembling somewhat the average human dietary were more cariogenic than a synthetic diet containing 67% sucrose. More recently, McClure ('52) reported production of caries in the rat on a diet deficient in a number of dietary essentials, although the sugar content was relatively low (18% cerelose).

Since our experiments definitely show a difference in cariogenicity related to constituents of the diet other than sugar, it remains to be determined what these constituents are and in what manner they make the teeth more or less resistant to dental caries. Investigations along this line are now in progress in our laboratories.

SUMMARY AND CONCLUSIONS

Litter mates of both albino rats and cotton rats were fed two synthetic diets that differed quantitatively in a considerable number of their constituents but were identical in their sugar content. One of these diets proved to be much more cariogenic than the other, indicating that the sugar content

of the diet does not necessarily provide a measure of its cariogenicity.

ACKNOWLEDGMENTS

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EFFECT OF VITAMIN B_{12} IN THE DIET OF THE RAT ON THE VITAMIN B_{12} CONTENTS OF MILK AND LIVERS OF YOUNG [1]

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Vitamin B_{12} has been shown to be essential for optimum reproduction and lactation in rats on purified casein diets (Dryden, Hartman and Cary, 51; Meyer, Thompson and Elvehjem, '51) and on all-vegetable diets (Schultze, '49, '50a, b; Halvorson and Schultze, '50; Emerson, Kummer and Zanetti, '50; Sure, '50; VanLandingham and Hare, '51; Lepkovsky, Borson, Bouthilet, Pencharz, Singman, Dimick and Robbins, '51). The effect of vitamin B_{12} on lactation in rats has been reported by Lepkovsky et al. ('51) and Dryden, Hartman and Cary ('52) to be due to an inadequate milk supply. On the other hand, Meyer et al. ('51) stated that the lactation performance is correlated with the level of vitamin B_{12} in the milk. Richardson, Witten and Couch ('51) found that the vitamin B_{12} content of weanling rat livers increased with increasing levels of vitamin B_{12} in the dam's diet.

The work reported in this paper was designed to study the effect of the vitamin B_{12} in the dam's diet: (1) on the level of the vitamin in the milk, (2) on the storage of vitamin B_{12} in the livers of newborn and weanling rats, (3) on the quantity of milk produced, and (4) on the reproduction and lactation performance as measured by various animal data.

[1] This work was supported in part by a grant from the Harry Snyder Memorial Fund.

EXPERIMENTAL

Animals and diets

The animals used were albino rats obtained from Sprague-Dawley, Inc. At 22 days of age 30 male and 60 female rats were placed in wire bottom cages and fed a stock diet of dog chow.[2] At three months of age the females were divided according to weight into 4 groups of 15 rats each. The rats on each diet were distributed at random throughout the batteries in groups of three rats per cage.

The basal diet used was composed of 60% soybean oil meal,[3] 25% sucrose, 9.8% hydrogenated cottonseed oil,[4] 1% succinylsulfathiazole,[5] 0.1% choline chloride, 0.1% DL-methionine,[6] and 4% salt mixture.[7] In addition the following vitamins were added per 100 gm of diet: 1 mg thiamine, 1 mg riboflavin, 5 mg calcium pantothenate, 5 mg niacin, 1 mg pyridoxine, 0.05 mg biotin, 5 mg 2-methyl-1,4-naphthoquinone, 0.5 mg folic acid and 25 mg alpha-tocopherol acetate. Vitamins A and D were supplied in oil,[8] two drops per animal per week. Each drop contained approximately 1,380 units of vitamin A and 195 units of vitamin D. Microbiological assays showed this diet to contain no vitamin B_{12} per se. The 4 diets employed were: (1) basal diet, (2) basal diet plus 0.2 μg vitamin B_{12} per 100 gm diet, (3) basal diet plus 20 μg vitamin B_{12} per 100 gm diet, (4) stock diet.

[2] G. L. F. Big Red Dog Food (Pellets), Cooperative G. L. F. Marketing Service, Inc., Canandaigua, New York.

[3] Fifty per cent protein, low fiber solvent-extracted soybean oil meal, obtained from Archer-Daniels-Midland Co., Minneapolis, Minnesota.

[4] Primex, hydrogenated cottonseed oil and soybean oil with an iodine value of about 75, manufactured by Procter and Gamble Co., Cincinnati, Ohio.

[5] Sulfasuxidine obtained from Sharp and Dohme, Inc., Glenolden, Pennsylvania.

[6] Kindly supplied by Dow Chemical Co., Midland, Michigan.

[7] Salt mixture contained 2 gm $Ca_3(PO_4)_2$, 0.8 gm NaCl, 0.872 gm NaH_2PO_4, 0.27 gm $FeSO_4 \cdot 7H_2O$, 17.8 mg $CuSO_4 \cdot 5H_2O$, 40 mg $MnSO_4 \cdot H_2O$ and 0.2 mg $CoCl_2 \cdot 6H_2O$ per 100 gm of diet.

[8] Percomorph Liver Oil, Abbott.

The females were mated at 140 days of age, after being on the experimental diets for 6 weeks. The male rats were maintained on dog chow throughout the experiment, except during the periods of mating, when they ate the diet of the females. Nineteen days after the beginning of mating the pregnant females were placed in individual cages with solid bottoms containing wood shavings as litter. The dam and young were weighed within 24 hours after parturition. Between 36 and 48 hours after birth the young were cut to 6, three males and three females wherever possible. Weight was used as the criterion for cutting, the heaviest of each sex being saved in each litter. The livers and curds from the stomachs were removed from the young destroyed, the samples from each litter being pooled and frozen for subsequent analysis.

The dam and young of each litter were weighed 21 days after parturition. The young were then removed from the dams and placed in a wire bottom cage without access to food for 15 to 18 hours, at the end of which time they were placed with the dam. All food was removed from this cage and the young allowed to nurse until their hunger was satisfied (about two hours). They were then sacrificed and the livers and curds from the stomachs removed and frozen.

After a three- to 4-week rest, the females were mated again. This procedure was repeated a third time. After the third reproduction period the dams were killed and their livers removed and frozen.

Determination of vitamin B_{12}

The vitamin B_{12} contents of rat liver and milk curd samples were determined by the microbiological assay of Peeler, Yacowitz and Norris ('49) using *Lactobacillus leichmannii* (American Type Culture Collection 4797). Initial studies were carried out to determine the best method of extracting the vitamin B_{12} from the samples. Various procedures were tried, including autoclaving for 30 minutes at 15 lb. pressure in pH 4.5 and 5.5 acetate buffer, and digestion with pancreatin in phos-

phate buffer (pH 7.0) for 20 hours at 37°C. In the case of rat liver, autoclaving at pH 4.5 gave the largest extraction of vitamin B_{12}, and with rat milk curd, autoclaving at pH 5.5 gave the highest results. The liver and curd were homogenized in a Potter-Elvehjem homogenizer before treatment. Several samples of liver and milk curd were assayed for riboside activity by the method of Hoffman et al. ('49). At the dilutions used this activity was negligible in each instance, and hence riboside activity was not determined routinely.

The vitamin B_{12} results are not presented as absolute values, but as relative ones which can be justly compared. The vitamin B_{12} contents of liver have been expressed on the basis of fresh weight of tissue, whereas the values for milk are based on total solids, since the samples used were curd and not liquid milk.

RESULTS AND DISCUSSION

Vitamin B_{12} data on two-day-old rats

The results obtained in the first lactation period on the vitamin B_{12} contents of livers and milk curds of two-day-old rats as affected by the dam's diet are presented in table 1. The data show that a high level of vitamin B_{12} in the dam's diet caused an increase in the vitamin in the livers of her young and in her milk. Analyses of variance (Snedecor, '46) were performed on the values for the vitamin B_{12} contents of livers and milk solids. From the standard errors and the "t" tests it was found that there was no difference in either set of data between the first two diet groups, but a highly significant increase in vitamin B_{12} concentration on the high level of vitamin B_{12} and stock diet in each case. Although the concentration of vitamin B_{12} was markedly increased in the milk of females on the high vitamin B_{12} diet, it is probable that the high level of the vitamin in the two-day-old livers from these young was the result of placental transfer largely, since the time of nursing was so short. The placental transfer of vitamin B_{12} has been shown to occur in the rat by Chow, Barrows and Ling ('51), using Co^{60}-tagged vitamin B_{12}.

Vitamin B_{12} data on weanling rats

The data presented in table 2 show the results obtained on 22-day-old rats. It is apparent that the vitamin B_{12} content of the diet of the dam affected the concentration of the vitamin in her milk, a reflection of which was evidenced in the vitamin B_{12} content of the livers of her young at weaning. If a comparison is made between the vitamin B_{12} content of the milk curd obtained from the two-day-old and 22-day-old

TABLE 1

Effect of dam's diet on the vitamin B_{12} contents of livers and milk curds from two-day-old rats

DIET	LITTERS USED FOR ANALYSES OF		AVERAGE TOTAL SOLIDS IN MILK CURD PER RAT	AVERAGE VITAMIN B_{12}[1] PER GM	
	Liver	Milk		Milk solids	Liver[2]
	no.		gm	mμg	mμg
1. Basal	7 (21)[3]	7 (21)[3]	0.0822	6.68	16.4 (4.17)[4]
2. 0.2 μg B_{12} per 100 gm	5 (12)	5 (13)	0.0846	6.91	20.5 (5.01)
3. 20 μg B_{12} per 100 gm	4 (13)	4 (13)	0.0830	119**[5]	160** (40.3)
4. Stock	6 (22)	5 (21)	0.0722	22.4**	56.2**(13.5)

[1] Assayed on pooled livers and milk curds from each litter.

[2] Values expressed on fresh weight basis.

[3] Numbers in parentheses represent total number of rats in litters.

[4] Numbers in parentheses represent the vitamin content of the average whole liver. The average liver weights for individual animals in all groups ranged between 0.24 and 0.25 gm.

[5] ** indicates significance at the 1% level when compared with the basal group.

rats, one observes that the concentration of vitamin B_{12} was higher in the milk during the early stages of lactation than at the end. This was true in each diet group, with the exception of the 0.2 μg vitamin B_{12} group, in which there was no difference. Collins, Harper, Schreiber and Elvehjem ('51) observed a higher vitamin B_{12} level in the colostrum of the cow and goat than in the regular milk; and Moinuddin, Pope, Phillips and Bohstedt ('51) reported similar results with the colostrum of ewes.

It is evident that the concentration of vitamin B_{12} in the milk of rats can be increased significantly by feeding the vitamin to the dam. Haq, Rusoff and Gelpi ('52) have reported, on the other hand, that a vitamin B_{12} supplement, when fed at a level equivalent to 3.3 mg of vitamin B_{12} per day to lactating dairy cows, did not bring about an increase in the level of the vitamin in the milk. However, Harper, Richard and Collins

TABLE 2

Effects of dam's diet on the vitamin B_{12} contents of livers and milk curds from weanling rats

DIET	YOUNG USED FOR ANALYSES OF		AVERAGE TOTAL SOLIDS IN MILK CURD PER RAT	AVERAGE VITAMIN B_{12} [1] PER GM	
	Milk	Liver		Milk solids	Liver [2]
	no.	no.	gm	mμg	mμg
1. Basal	38 (8)[3]	54 (10)[3]	0.4693	3.62	10.9 (16.5)[4]
2. 0.2 μg B_{12} per 100 gm	40 (8)	42 (8)	0.3886	6.43	22.5 (33.7)
3. 20 μg B_{12} per 100 gm	41 (8)	52 (9)	0.4382	73.2**[5]	304**(456)
4. Stock	43 (10)	41 (10)	0.7300	11.6	137**(262)

[1] Assayed on individual young in each litter.

[2] Values expressed on fresh weight basis.

[3] Numbers in parentheses represent the number of litters.

[4] Numbers in parentheses represent the vitamin content of the average whole liver. The average liver weights of the young on these diets were: diet 1, 1.62 gm; diet 2, 1.53 gm; diet 3, 1.56 gm; diet 4, 2.02 gm.

[5] ** indicates significance at the 1% level when compared with the basal group.

('51) found that cobalt fed to ewes on a diet containing adequate cobalt caused an increase in the vitamin B_{12} level in the milk.

The comparison of the liver vitamin B_{12} contents in the two age groups is also interesting, although it must be remembered that the values obtained on the livers of two-day-old rats may not be representative, since these rats were the lightest in weight in each litter. Weanling rats from dams on the basal diet had a lower concentration of vitamin B_{12} in their livers than their litter mates at two days of age. This

may indicate a depletion of vitamin B_{12} stores obtained during fetal life, caused by a limited amount of the vitamin received from the dam's milk during the lactation period. Young from dams receiving 0.2 μg of vitamin B_{12} per 100 gm of diet showed no difference in the vitamin B_{12} concentration in the liver at two and 22 days of age; in addition, the vitamin B_{12} contents of the respective milks were also the same. Rats receiving 20 μg % of vitamin B_{12} and those on the stock diet passed sufficient vitamin B_{12} into the milk to cause an increased storage of the vitamin in the livers of young by the time of weaning. Actually, the concentration of vitamin B_{12} in the milk of the dams on the higher levels of vitamin B_{12} decreased in this period, but the consumption of a high level of vitamin B_{12} gave more than was needed for growth, so storage occurred.

Animal data

A summary of the animal results for the first reproduction-lactation period is presented in table 3. The data show that vitamin B_{12} at the higher level (20 μg %) very significantly increased both the birth weights and the weaning weights of the young from dams on this diet over similar weights of young from dams on the basal diet. The effect of the lower level of vitamin B_{12} on the average birth weight was significant at the 5% level. There was no difference in birth weights when comparing the young from dams on the stock diet and the young from dams on the high level of vitamin B_{12}. The average weaning weights of rats from females on the basal diet and the low level of vitamin B_{12} were not statistically different, but the average weaning weights of young from stock diet females and the high vitamin B_{12} group were each highly significantly greater than the average weaning weights of young from females on the basal diet. However, the average weaning weight of young from stock diet females was very significantly greater than that of young from dams receiving the high level of vitamin B_{12}.

There was no difference in the mean weight change of the dams on the different diets during lactation; there was an average increase in weight of 15 to 26 gm. Differential white cell counts showed no abnormality due to a vitamin B_{12} deficiency.

The quantity of milk produced may have some bearing on the differences in weaning weights between the young on all experimental diets and those on the stock diet. In table 4 are

TABLE 3

Effect of vitamin B_{12} content of diet on reproduction and lactation in the rat as measured by animal criteria

DIET	TOTAL YOUNG	AVERAGE YOUNG BORN PER LITTER	AVERAGE WEANED PER LITTER [1]	AVERAGE BIRTH WEIGHT [2]	AVERAGE WEANING WEIGHT [2]
	no.	no.	no.	gm	gm
1. Basal	82 (10)[4]	8.2	5.4	5.59	30.5
2. 0.2 μg B_{12} per 100 gm	65 (8)	8.1	5.3	5.77*[5]	32.1
3. 20 μg B_{12} per 100 gm	72 (9)	8.0	5.8	6.02**	33.8**
4. Stock	83 (10)	8.3	4.3	5.91**	42.4**

[1] Litters cut to 6 animals 36 to 48 hours after parturition.

[2] Average weight of animals surviving to weaning.

[3] At 21 days of age.

[4] Numbers in parentheses represent number of litters.

[5] * indicates significance at the 5% level, ** significance at 1% level when compared with basal group.

presented data on the amounts of milk removed from the stomachs of young from females on the various diets for all three lactation periods. All three periods were used since there were no differences among them as far as amounts of milk produced were concerned. It is not suggested that the milk in the stomachs of the young represented the total quantity of milk produced by a dam. However, the results on one diet can be compared with those on another, since great care was taken to remove the curd in the same manner from the stom-

achs of all young. The data show that the quantity of milk consumed by the young of females on the stock diet was significantly greater than that obtained by young of females on any of the experimental diets. Thus, vitamin B_{12} does not appear to be concerned with increasing milk yield under these experimental conditions, if it is permissible to consider milk consumption by the young as a measure of milk yield. This is contrary to the findings of Lepkovsky et al. ('51) and Dryden, Hartman and Cary ('52). It is possible, however, that

TABLE 4

Effect of vitamin B_{12} content of dam's diet on quantity of milk removed from stomachs of weanling rats

DIET	FEMALES [1]	TOTAL YOUNG	AVERAGE YOUNG PER LITTER	AVERAGE AMOUNT OF MILK EXPRESSED AS TOTAL SOLIDS	
				Per female	Per young rat
	no.	no.	no.	gm	gm
1. Basal	10	54	5.4	2.4677	0.4570
2. 0.2 μg B_{12} per 100 gm	8	44	5.5	2.3302	0.4237
3. 20 μg B_{12} per 100 gm	9	52	5.8	2.6071	0.4512
4. Stock	14	74	5.3	3.4581** [2]	0.6542**

[1] The only females considered were those weaning 5 or 6 young. Results include data from three lactation periods.

[2] ** indicates significance at the 1% level when compared with the basal group.

on this diet some other factor was limiting and thus prevented vitamin B_{12} from exercising its influence on milk production.

During the prolonged feeding of this all-vegetable diet it is believed that a deficiency of some factor other than vitamin B_{12} occurred in the females. The results of the second and third reproduction-lactation periods are not sufficiently extensive to warrant any broad generalizations. However, by the third breeding period only three or 4 females of 14 bred on each experimental diet cast litters, and of those young cast

there was a high percentage born dead (55%). Of the stock females 8 out of 13 bred cast young with only 7% born dead, and 90% of the 6 animals per litter were weaned. There seemed to be no relationship between the vitamin B_{12} level in the dam's diet and the poor reproduction and lactation performance in the second and third matings.

The diet as listed is thought to be complete in all known nutrients for the rat. The protein level of the diet was 30% and all of the essential amino acids were present in amounts calculated to be adequate for reproduction and lactation. The fat was supplied as hydrogenated cottonseed oil (9.8%) which, according to the manufacturer,[9] contained approximately 6% linoleic acid. The soybean oil meal contained 1% fat, and on the basis of an iodine value of 133 for soybean oil, the soybean oil meal contributed about 300 mg of linoleic acid per 100 gm of diet (Bailey, '51). On the basis of a feed consumption of 10 gm per day, a rat would receive approximately 90 mg of linoleic acid daily. According to Quackenbush et al. ('42), the daily consumption of 100 mg of ethyl linoleate three weeks before breeding was sufficient for normal reproduction and lactation, although these rats were maintained to maturity on a fat-free diet. Recently, Kummerow, Pan and Hickman ('52) reported that 5% hydrogenated oil[10] was adequate for reproduction but not for lactation in rats. None of the young was weaned. It is possible, therefore, that a deficiency of essential fatty acid developed as the length of time on the experimental diet increased. There were, however, no gross signs of essential fatty acid deficiency in the dams.

The levels of certain B vitamins were raised because of the inclusion of sulfasuxidine in the diet. Little work, however, has been done on the effect of vitamin B_{12} on reproduction with sulfonamides in the diet. Schultze ('50a) obtained better reproduction in rats on a soybean oil meal diet plus phthalylsulfathiazole than without it, and observed no cases of acute uremia of the newborn. He suggested that there was

[9] See footnote 4, p. 276.

[10] Crisco.

an increased intestinal synthesis of vitamin B_{12} caused by the alteration of the flora. Bosshardt, Huff, Paul and Barnes ('49), using mice, found that sulfasuxidine retarded growth on purified diets, a retardation which could not be corrected by biotin and folic acid but was partially overcome by a vitamin B_{12} concentrate and completely prevented by a crude liver preparation. Jones, Rogers and Stone ('49), using purified diets with rats, observed retarded growth when sulfasuxidine was included. This could be prevented by purified liver extract, which contains vitamin B_{12} and probably other factors.

It is possible that a diet containing sulfasuxidine, and soybean oil meal as the only source of protein, is deficient in some factor other than vitamin B_{12}. That there may be other unknown factors concerned with growth, reproduction and lactation in rats has been suggested by several groups of workers. Piccioni, Rabbi and Moruzzi ('51) stated that small amounts of whole cow's milk, and not vitamin B_{12}, gave good reproduction on a purified casein diet. Betheil and Lardy ('49) found antipernicious anemia liver extracts to be partially effective in overcoming the growth depression of young rats fed thyroid powder, whereas whole liver gave a greater response. The factor was also present in fish solubles, tomatoes and yeast. Ershoff ('50) prolonged the survival of hyperthyroid rats by feeding yeast or whole liver. Stern and McGinnis ('50), using a soybean oil meal diet, obtained low weaning weights of rats, which were not affected by vitamin B_{12} but markedly influenced by streptomycin and aureomycin. Lepkovsky et al. ('51) reported that the "impairment of the lactation of first litter females is caused by unknown factors inherent in the vitamin B_{12} deficient ration, but vitamin B_{12} deficiency is not the major cause." Halvorson and Schultze ('50) found no evidence of uremia of newborn rats when soybean protein was purified, although before purification a high incidence had occurred which could be prevented by vitamin B_{12} injections. The explanation of these results is unknown, although the occurrence of uremia in newborn rats would not

seem to be solely a result of vitamin B_{12} deficiency, since the method of purification of the soybean protein would have removed the small amount of vitamin B_{12} that was present.

At the end of the third lactation period the females were killed and vitamin B_{12} analyses made on their livers. These results are presented in table 5. It is evident that the livers from females on the basal diet contained about half as much vitamin B_{12} as those from females on the stock diet. Increasing the level of vitamin B_{12} in the diet caused an increase in the concentration of the vitamin in the livers of females consuming

TABLE 5

Vitamin B_{12} content of dams' livers

DIET	FEMALES	AVERAGE WEIGHT OF DAMS [1]	AVERAGE WEIGHT OF LIVERS	AVE. VITAMIN B_{12} [2]
	no.	gm	gm	mμg per gm
1. Basal	11	265	10.9	66.5 (718)[3]
2. 0.2 μg B_{12} per 100 gm	13	280	11.1	117* [4] (1269)
3. 20 μg B_{12} per 100 gm	14	281	11.2	127** (1394)
4. Stock	13	297	11.3	145** (1600)

[1] Sacrificed after third lactation, when they were 13 months old.

[2] Values expressed on fresh weight basis.

[3] Values in parentheses indicate average millimicrograms of vitamin B_{12} per liver.

[4] * indicates significance at the 5% level, ** significance at the 1% level when compared with basal group.

the diet. There was no statistical difference among the values for livers from females receiving additional vitamin B_{12} and the stock diet. It is interesting to note that the weanling rats from females on the high vitamin B_{12} diet had more of the vitamin in their livers than the young from stock animals, although the females did not store any more than the stock diet females. The excess dietary vitamin B_{12} seemed to be passed on to the young rather than stored in the liver of the dam.

SUMMARY

1. Vitamin B_{12} in the diet of the dam was transferred to the young during prenatal life, as indicated by the level of

the vitamin in the livers of two-day-old rats. The amount found in the livers of young from females on a high vitamin B_{12} diet was 10 times the amount found in the livers of young from females on the basal diet.

2. The level of vitamin B_{12} in the milk varied directly with the level in the diet of the dam. The milk from dams on all diets but one contained a higher concentration of vitamin B_{12} two days after parturition than at the end of the lactation period.

3. The concentration of vitamin B_{12} in the livers of 22-day-old young from females on the high vitamin B_{12} diet and from stock females was greater than the concentration of the vitamin in livers of litter mates sacrificed at two days of age. In the case of the basal young, there was a decrease over the lactation period in the amount stored in the liver.

4. The birth weights of young varied directly with the level of vitamin B_{12} in the female's diet. A high level of vitamin B_{12} caused an increase in weaning weight, but not as much as the feeding of the stock diet.

5. The quantity of milk curd extracted from the stomachs of weanling young from dams on the stock diet was significantly higher than that removed from the young of dams fed any of the experimental diets.

6. Results of the second and third reproduction-lactation periods indicated a poor reproductive performance of females on the experimental diets, as shown by few conceptions, many young born dead and few weaned as compared with the stock diet animals. The possibility of a deficiency of an unknown factor developing due to the prolonged feeding of the all-vegetable diet plus sulfasuxidine has been discussed.

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AN UNIDENTIFIED FACTOR IN PEANUT MEAL REQUIRED BY THE CHICK[1]

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ONE FIGURE

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In the course of work designed to study the growth-promoting properties of certain natural materials, a diet was developed which was believed adequate in all known nutrients necessary for the growth of chicks. The diet consisted principally of cornstarch and purified casein, supplemented with methionine, glycine, the required known vitamins and essential minerals. In preliminary work, it was found that the diet was markedly deficient in arginine and that the addition of this amino acid resulted in greatly improved growth and vigor. However, even when the diet contained high levels of supplementary arginine, it did not permit an optimum rate of growth.

It is the purpose of this paper to present results which show that an unidentified factor is lacking in the casein-starch diet, and that the factor is furnished by alcohol-extracted peanut meal.

EXPERIMENTAL

After the initial observation that a diet containing 25% purified casein did not support optimum growth of chicks,

[1] This work was supported in part by a grant from the International Minerals and Chemical Corporation, Chicago, Illinois, and was conducted in the Nutrition Laboratories of the Department of Poultry Husbandry.

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other supplies of casein were examined. Casein from two sources was obtained and fed in both the crude and purified forms. Arginine HCl was added to each diet to bring the total arginine content to 0.96%. In addition, two lots of chicks received the basal diet to which 5 and 10% alcohol-extracted peanut meal [3] were added, with a corresponding reduction of casein to 22 and 19% and appropriate adjustments in the percentage of cornstarch.

Neither source of casein, whether crude or purified, supported optimum growth of chicks. The inclusion of 5 or 10% alcohol-extracted peanut meal in the diet resulted in average chick weights of 241 and 306 gm, respectively, at 4 weeks of age, as compared to 150 gm for chicks reared on the diet containing 25% casein. Three experiments were then conducted to determine if the growth response from added peanut meal was due to the arginine or to an unidentified factor which the meal provided.

Cross-bred (R.I.R. $\times$ B.P.R.) male day-old chicks were used in all experiments. At the beginning of each experiment the chicks were divided into equal weight groups of 15 chicks per lot. A total of 41 lots was used in the investigation. The chicks were maintained in electrically heated battery brooders with feed and water allowed ad libitum during the experimental period. Individual chicks were weighed at weekly intervals.

The composition of the basal diet fed the chicks is given in table 1. All the vitamins were in excess of the reported requirements except niacin, which was supplied at the level of 1.78 mg per 100 gm of diet as recommended by the Committee on Animal Nutrition of the National Research Council ('50). However, in a separate experiment to determine the adequacy of the diet in niacin, no growth response was obtained when niacin was added at levels equivalent to those supplied by the peanut meal. Furthermore, Norris and Chin ('52) found that the niacin requirement for fast-growing New

[3] Continuously extracted with ethanol for 48 hours, followed by methanol extraction for 24 hours.

Hampshire chicks fed a purified diet was between 0.89 and 1.78 mg per 100 gm of diet.

At a preliminary report of this work at the annual meeting of the Poultry Science Association in 1952, it was suggested that the biotin content of the diet was not adequate to meet

TABLE 1

Basal diet

CONSTITUENTS	AMOUNT	CONSTITUENTS	AMOUNT
	gm		*mg*
Purified casein [1]	25.0	Alpha-tocopherol acetate	5.5
Cornstarch	64.4	Menadione	0.22
Hydrogenated vegetable fat (Primex)	2.5	Thiamine HCl	0.33
		Riboflavin	0.55
Ground cellophane	3.0	Niacin	1.78
Iodized salt	0.5	Calcium pantothenate	1.65
Dicalcium phosphate	2.0	Pyridoxine HCl	0.55
Limestone	1.0	Para-aminobenzoic acid	11.0
Choline	0.15	Inositol	22.0
KCl	0.42	Biotin	0.011
$MgSO_4$	0.25		*µg*
	mg	Vitamin B_{12}	3.0
$MnSO_4 \cdot 4H_2O$	22.0	Folacin	400.0
$FeSO_4 \cdot 7H_2O$	10.0	Vitamins A and D_3 [2]	
$CuSO_4 \cdot 7H_2O$	1.1		
$ZnCl_2$	1.1		
$CoCl_2 \cdot 6H_2O$	0.14		

[1] Purification consisted of 5 washings over a 48-hour period with an acid salt solution (pH 2.5) at a maximum temperature of 60°C. followed by a final washing with water at pH 4.7 and oven drying at 60–70°C.

[2] Vitamin A palmitate and irradiated animal sterols in cottonseed oil, added to the ration to supply 2,000 I.U. of vitamin A and 180 A.O.A.C. units of vitamin D_3 per pound of feed.

the chick's requirement. However, when the diet was supplemented with 0.015 and 0.02 mg of biotin per 100 gm, no increase in growth was obtained (Norris and Dam, '52).

In each experiment 5, 10 and 15% of extracted peanut meal were added to mixtures of the basal diet and the percentages of casein were reduced to 22, 19 and 15.5%, respectively. The total crude protein in the diet was thus maintained at 23%.

Inasmuch as the added peanut meal exceeded the quantity of casein withdrawn, the percentage of cornstarch was reduced by the appropriate amount. Supplementary arginine was added to the basal diet at levels equal to or higher than those contributed by the peanut meal. The diets containing peanut meal were also supplemented with arginine at several levels.

The purified casein and extracted peanut meal were each assayed microbiologically for 10 of the amino acids considered essential for the chick, and for tyrosine. The arginine content of the purified casein was found to be 3.45%, and that of the peanut meal 5.84%. The assay values plus the values reported for glycine, glutamic acid and cystine by Block and Bolling ('51) were used to calculate the amino acid content of each diet. Methionine and glycine were added to each diet in such amounts that the total methionine plus cystine was maintained at 0.9% and the total glycine was maintained at 1.2% of the diet, regardless of the amount of peanut meal supplied. Supplementary arginine was supplied in the form of the monohydrochloride.

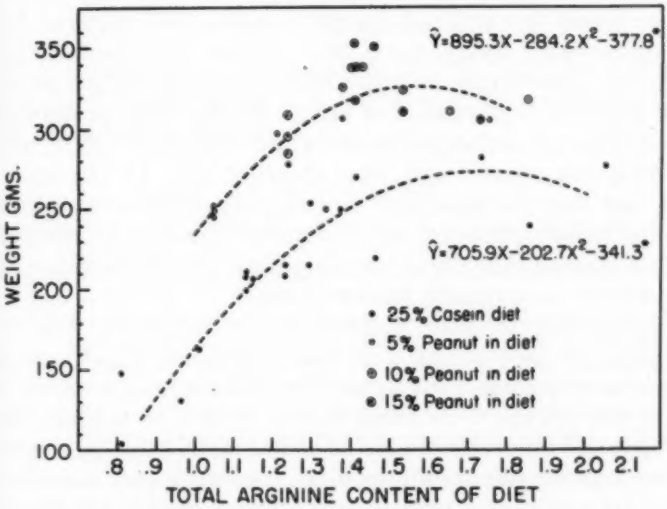
RESULTS

The results of the three experiments are summarized in figure 1 by plotting the average 4-week weights of each lot against the total arginine content of the diet. In all cases the inclusion of peanut meal in the ration resulted in a marked growth response over that obtained with the rations containing casein and levels of arginine equivalent to or higher than those provided by the peanut meal.

After the adjustment of the means for the curvilinear regression due to increasing levels of arginine (Snedecor, '48), the difference between the mean of the rations containing casein supplemented with various levels of arginine and the rations containing peanut meal providing equivalent levels of arginine shows a highly significant F value.[4]

[4] $F = 34.6$ with 38 degrees of freedom for error. Tabular F value $= 7.35$ at $P = 0.01$.

Maximum growth was obtained with rations containing 15% peanut meal or with rations containing 5 or 10% peanut meal plus sufficient arginine HCl to provide a total of approximately 1.4% arginine in the diet. The average weight of 7 lots of chicks receiving 1.38 and 1.41% arginine and either 5, 10, or 15% peanut meal was 331 gm, as contrasted with an average weight of 247 gm for three lots of chicks



* The broken lines represent the results of the respective quadratic equations calculated from the data by curvilinear regression.

Fig. 1 Growth of chicks supplied various levels of arginine in a purified diet and a similar diet containing peanut meal.

receiving between 1.37 and 1.47% arginine but not peanut meal.

Klose et al. ('38) suggested that the arginine in casein may be partially unavailable for the chick. However, supplementation of the casein diet with the same amount of arginine as contributed by 5, 10, and 15% peanut meal, respectively, failed to support growth comparable to that obtained on diets containing peanut meal. It does not appear, therefore, that the

peanut meal promoted growth by supplying a more available form of arginine.

DISCUSSION

Almquist ('47) reported that the chick's requirement for arginine is 1.2% of the diet. Later, Almquist and Merritt ('50) presented data showing that the arginine requirement of chicks is approximately 6% of the total dietary protein. They found that chicks fed a 20% protein diet required 1.2% arginine, while chicks fed a 30% protein diet required 1.8% arginine. According to Almquist and Merritt's ('50) results, the arginine requirement of chicks fed the 23% protein diet used in the present experiments is approximately 1.38%, which corresponds closely with that observed (fig. 1). In view of the fact that the basal diet was supplemented with an excess of the known vitamins, or an amount found to be adequate, it is apparent that the extracted peanut meal did not increase growth by supplying a known vitamin.

Except for arginine, methionine, cystine and glycine, the amount of each amino acid was decreased slightly as the amount of peanut meal in the diet was increased. Since the observed effects were found not to be due to arginine deficiency, and the levels of glycine and of methionine plus cystine were kept constant in all the diets, it seems highly improbable that the addition of peanut meal affected growth by changing the amino acid balance. Therefore, it appears that under the experimental conditions peanut meal supplied an unidentified factor necessary for optimum growth of chicks.

SUMMARY

The addition of alcohol-extracted peanut meal to a purified chick diet containing purified casein as the source of protein resulted in a growth response which was only partially accounted for by its arginine content. The response also appeared not to involve any deficiency of known vitamins required by the chick.

Under the conditions of this experiment, therefore, alcohol-extracted peanut meal supplied an unknown factor needed for chick growth which was lacking in the purified diet.

The results also indicated that the chick's requirement for arginine is approximately 1.38% of a diet containing 23% protein. This confirms the work of Almquist and Merritt ('50), who found the arginine requirement to be approximately 6% of the total dietary protein.

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